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16. Abstract (MAXIMUM 200 WORDS)

A workshop was held 6 – 16 January 2008 to evaluate the ability of various analysis methods to detect, enumerate, and determine the viability of phytoplankton at concentrations to be expected after treatment by successful ballast water treatment systems. Researchers from Woods Hole Oceanographic Institute, Moss Landing Marine Laboratory, Naval Research Laboratory, and Fluid Imaging Technologies were provided with six samples with known concentrations of live and dead *Tetraselmis* species in prepared seawater. A seventh sample contained live *Tetraselmis* plus dissolved organic matter and mineral matter to better mimic actual ballast water samples. Methods investigated included manual microscopy with vital stains, microscopy of concentrated samples stained with different stains, flow cytometry of stained organisms, pulsed amplitude modulated (PAM) fluorometry, and an enhanced flow-through system with imaging capability. Statistical analyses were performed on the results. Overall the methods were all self-consistent. Although there were differences from sample to sample, comparisons showed no real differences could be established indicating one method is better than others. Advantages and disadvantages of each analytical method for routine use are provided.

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EXECUTIVE SUMMARY

Ballast water is an inexpensive and efficient means for ships to maintain trim, counteract list, and adjust waterlines in response to cargo loading and unloading or in response to foul weather. Ballast water is taken aboard, stored in tanks, moved about, and discharged as required. In addition to the water itself, ballast water contains organisms that were taken on board with the water. The ballast water volume for any given ship can be tremendous – hundreds to hundreds of thousands of metric tons. Frequently, ships' tanks will be ballasted in one port and deballasted in another. The entrained organisms are generally discharged along with the ballast water. This process has been identified as a vector for the translocation of non-indigenous species (NIS).

The United States Coast Guard (USCG) and the International Maritime Organization (IMO), which governs international maritime law, have promulgated regulations designed to reduce the transport and transfer of NIS by ships' ballast water. More stringent regulations for ballast water treatment are anticipated from IMO, USCG, and possibly other domestic regulators in the near future. These regulations will likely result in the need for on-board ballast water treatment to kill, remove or otherwise inactivate organisms in the ballast water. Ballast water treatment equipment (BWTE) must be tested and approved for use before it can be generally installed and used aboard ships. In order to show that ballast water discharge is in compliance with these stringent regulations, measurement methods need to be developed and validated that can sufficiently characterize the expected low concentrations of organisms within treated ballast water. The same methods are also required for compliance testing after systems are used aboard ships.

In order to ensure that BWTE is stringently tested before granting approval, USCG has partnered with the Environmental Protection Agency's Environmental Technology Verification (ETV) Program to develop protocols for testing BWTE. IMO has likewise specified standardized protocols through their Guidelines. The protocols call for characterization of basic water quality parameters and assessment of concentrations and viability of ambient and experimentally added test phytoplankton and zooplankton before and after treatment. Methods must be developed and validated for determining the concentration and viability of planktonic species over the concentration ranges that are normally encountered in support of BWTE evaluations.

The Ballast Water Treatment Test Facility (BWTTF) at the Naval Research Laboratory in Key West, Florida (NRLKW) is used to evaluate the efficacy of BWTE according to standardized protocols including those established in the ETV Program and the IMO's G8 Guidelines. This unique facility's primary objective is to provide third-party, independent and objective testing of BWTE and to provide data from standardized tests to other entities for certification (for example, USCG approval). Another primary objective of this facility is to develop and disseminate methods and procedures that can be used by other third-party test facilities during their evaluations of BWTE to ensure that results from various facilities performing standardized testing can be compared.

NRLKW has conducted tests of full-scale BWTE according to the standardized protocol. During that testing significant man-hours were expended determining the concentrations and viability of phytoplankton and zooplankton in challenge and post-treatment water samples. It is impractical to characterize the full volume of treated ballast water, thus sub-sampling and analysis methods are necessary to determine sample concentrations and viability. Manual microscopic analysis methods used in support of the BWTTF allowed three 1-mL samples to be analyzed in approximately 4.5 hours. As biological samples can deteriorate with

time (e.g., cells can die), it is impractical to increase the number of subsamples used to determine sample concentrations and viabilities. Further, as a result of a small number of subsamples and the low concentrations of viable organisms in post-treatment samples, it is difficult to determine with statistical certainty that the full volume of treated ballast water meets the stringent requirements for viable phytoplankton. These same types of difficulties are anticipated when samples of ship's ballast water discharge are analyzed to ensure compliance with discharge regulations.

In order to explore the current issues and limitations in determining low concentration levels of phytoplankton in treated ballast water samples, the USCG R&D Center tasked NRLKW to conduct a "Phytoplankton Enumeration and Evaluation Workshop" at the BWTTF in Key West, FL. This workshop was conducted from January 6 to January 16, 2008 and brought together four teams for evaluating a variety of samples containing live and dead phytoplankton.

The four teams and their methodological approaches were:

- **NRLKW** – Manual microscopy using an epifluorescence microscope and Sedgwick Rafter counting cells. DNA stains were used to identify non-viable cells.
- **Woods Hole Oceanographic Institute (WHOI)** – Sample concentration using filtering combined with manual microscopy on the filters using an epifluorescence microscope. DNA and vital stains were used to identify viable and non-viable cells.
- **California State University's Moss Landing Marine Laboratory (MLML)** – Flow Cytometry using DNA and vital stains to identify viable and non-viable cells. Pulsed Amplitude Modulated (PAM) Fluorometry, cell culturing and biochemical analyses were also used. Note that only the workshop samples MLML analyzed using Flow Cytometry and DNA and vital stains are reported here, and the method recommendation below pertains only to this method.
- **NRLKW/Fluid Imaging Technologies** – Updated FlowCAM® (an imaging flow cytometer manufactured and sold by Fluid Imaging Technologies, Inc. [Yarmouth, Maine]) and DNA stains to identify non-viable cells.

Prior to the start of the workshop, NRLKW developed methods to generate representative samples of live and dead phytoplankton (*Tetraselmis* sp., a flagellate) concentrations as well as various other substances such as test dust and dissolved solids that are requirements in BWTE evaluations. Because the concentration of the prepared samples were known only approximately, it was not possible to determine the accuracy of the teams' methods. These samples did, however, allow for an assessment of the repeatability and consistency of the various methods used for cell enumeration and viability classification.

During the workshop, the teams analyzed seven separate, complex phytoplankton samples. Each team was provided with a sample with an approximately known concentration of live and dead *Tetraselmis* and of sufficient volume that subsamples could be taken as appropriate. The teams analyzed the subsamples according to their method's protocol and reported their results for all subsamples analyzed. Results were compared on a replicate by replicate basis for each method, and means were compared across methods. Overall the results of the different methods were within a factor of two or four of the expected live concentration. There was greater variability for counts of non-viable cells. Statistical analyses performed on the reported results indicate that although there were significant differences between the techniques, no trend could be established. As such, all of these methods are recommended for phytoplankton analysis.

Phytoplankton Enumeration and Evaluation Experiments

In addition to reporting the direct results of the workshop experiments, the report describes the methods used in general terms and then provides detailed instructions in an appendix. The advantages and disadvantages of each method are given. Cautions related to specific methods or general testing are provided. Specific information and caveats are provided for the commercially available FlowCAM® imaging flow cytometer.

Recommendations include investigations to determine appropriate stains and concentrations for phytoplankton enumeration and evaluation. To ensure reliable results during BWTE testing, it is highly recommended that facilities evaluating BWTE apply at least two of these techniques when performing phytoplankton analyses.

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LIST OF ACRONYMS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BWTE	Ballast water treatment equipment
BWTTF	Ballast water treatment test facility
C	Carbon
CH	Channel
CMAC	7-amino-4-chloromethylcoumarin
CMFDA	5-chloromethylfluorescein diacetate
DF	Degrees of freedom
DMSO	Anhydrous dimethylsulfoxide
DOC	Dissolved organic carbon
DSP	Digital signal processor
EPA	Environmental Protection Agency
ESD	Equivalent spherical diameter
ETV	Environmental Technology Verification
FDA	Fluorescein diacetate
FSW	Filtered seawater
GUI	Graphical user interface
IMO	International Maritime Organization
ISO	International Standards Organization
L	Liter
LED	Light emitting diode
mg	Milligram
min	Minute
mL	Milliliter
MLML	Moss Landing Marine Laboratories
mm	Millimeter
mM	Millimolar
MM	Mineral matter
MPN	Most probable number
MSE	Mean square error
NIS	Non-indigenous species
NRL	Naval Research Laboratory
NRLKW	Naval Research Laboratory, Key West, Florida
PAM	Pulsed amplitude modulated (fluorometry)
PC	Personal computer
POM	Particulate organic matter
psu	Practical salinity units
SR	Sedgwick Rafter
SS	Subsample
TSS	Total suspended solids
TOC	Total organic carbon
US	United States
USCG	United States Coast Guard
µg	Microgram

LIST OF ACRONYMS (Continued)

µL	Microliter
UV	Ultra violet
WHOI	Woods Hole Oceanographic Institution

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1 BACKGROUND AND INTRODUCTION

Nearly all commercial and military ships and some recreational vessels carry ballast water onboard. The ballast water is stored in tanks aboard the ship and serves a variety of purposes including management of ship trim and list as well as adjusting ship waterline in response to the on-loading and off-loading of cargo or as a function of ambient weather conditions. Ballast water volumes are, by necessity, very large (hundreds to hundreds of thousands of metric tons). Frequently, ships' tanks will be ballasted in one port and deballasted in another. This process has been identified as a vector for the translocation of non-indigenous species (NIS) and it is currently regulated by the United States Coast Guard (USCG). More stringent regulations are anticipated for ballast water treatment by the International Maritime Organization (IMO), which governs international maritime law, USCG, and possibly other domestic regulators in the near future. These regulations are expected to result in the need for on-board ballast water treatment to kill, remove, or otherwise inactivate organisms within ballast water.

With support from and in partnership with the USCG, the Naval Research Laboratory Key West (NRLKW) has designed, constructed and operates a prototype Ballast Water Treatment Test Facility (BWTTF). The BWTTF is used to evaluate the efficacy of Ballast Water Treatment Equipment (BWTE) according to standardized protocols. This is a unique facility with the objective of providing third-party, independent and objective testing of BWTE and providing data from standardized shore-based tests to other entities for approval and certification. Another major objective of this facility is to develop and disseminate methods and procedures that can be used by other third-party test facilities during their evaluations of BWTE to ensure that results from various facilities performing standardized testing are comparable.

Standardized shore-based testing of BWTE necessitates complete characterization of the challenge water used to evaluate the efficacy of the equipment. Characterization of basic water quality parameters such as pH, total dissolved solids, salinity and others will validate compliance with water chemistry parameters that are defined in standardized test protocols. To document biological treatment performance, concentrations and viability of both indigenous (ambient) and experimentally added phytoplankton and zooplankton must be determined before and after treatment. It is likely that more stringent regulations under consideration by the USCG, as well as those being considered by individual States and Countries, will make it increasingly difficult to ensure that treated water is compliant with these regulations using the currently available methods and analysis techniques.

When characterizing ballast water samples, it is currently impractical to characterize the full volume of the treated ballast water. Thus smaller samples and subsamples are analyzed to estimate sample phytoplankton concentrations and assess viability. Manual microscopic analysis methods that were used in support of early BWTE evaluations at the BWTTF allowed only three 1-mL samples to be analyzed in approximately 4.5 hours. Samples deteriorate with time (e.g., cells can die); previous work at the BWTTF indicated 6 hours before samples deteriorated beyond usefulness. Operator fatigue can also affect accurate analysis. Thus it is impractical to increase the number of subsamples used to determine sample concentrations and viability. These considerations make it difficult to estimate with a high degree of statistical confidence that treated ballast water meets the stringent requirements for viable phytoplankton following treatment.

To explore the current issues and limitations in quantifying low concentrations of phytoplankton in treated ballast water samples, the USCG R&D Center tasked NRLKW to conduct a "Phytoplankton Enumeration and Evaluation Workshop" at the BWTTF in Key West, FL. A survey of potential participants in the

summer of 2007 identified three organizations in addition to NRLKW that were interested in participating in the workshop.

In November 2007, a detailed description of the type of work that would be performed at the workshop was provided to the potential participants (Appendix A). These organizations were asked to provide NRLKW with a technical and cost proposal for participating in the workshop to perform the tasks described. Essentially, each of the groups was to conduct analysis on phytoplankton samples to determine their concentration and viability. The flagellate *Tetraselmis* was to be used as the phytoplankton surrogate in the samples. The concentration ranges and Dead:Live ratios for the samples to be analyzed in support of the workshop are summarized in Table 1.

Table 1. Projected concentrations and dead:live ratios for phytoplankton samples.

Concentrations	Dead:Live Ratio	Dead:Live Ratio	Dead:Live Ratio
1 mL^{-1}	0:1	10:1	100:1
10 mL^{-1}	0:1	10:1	100:1
100 mL^{-1}	0:1	X	X

If time allowed, additional measurements would be made on complex samples more representative of the ballast water samples associated with BWTE evaluations at the BWTTF. That is, in addition to the single phytoplankton surrogate, sediments and dissolved organic matter would be added to the samples in the concentration ranges and with the Dead:Live ratios represented in Table 1. Adding these materials would simulate the lower concentrations of dissolved organic carbon, particulate carbon, and mineral matter consistent with metrics used during standardized ETV testing (i.e., 4 – 6 mg L^{-1} dissolved organic carbon; 4 – 6 mg L^{-1} particulate carbon; 8-11 mg L^{-1} mineral matter).

Proposals were received from the three organizations for supporting the phytoplankton enumeration experiments to be performed at NRLKW from January 6 - 16, 2008. Two of the groups, the Woods Hole Oceanographic Institute (WHOI) and California State University's Moss Landing Marine Laboratory (MLML) proposed to support the phytoplankton enumeration experiments using measurement techniques independently developed in their laboratories.

The third group was Fluid Imaging Technologies, Inc., a small business that manufactures the FlowCAM®, an imaging, flow-cytometer based system. Fluid Imaging proposed to support the workshop in conjunction with NRLKW and proposed to upgrade an NRLKW-owned FlowCAM® system to their most current and advanced configuration (one with a digital signal processor (DSP)) and to provide NRLKW personnel training on its use. Fluid Imaging also agreed to provide support during the first few days of testing at Key West to assist NRLKW in development of the final measurement protocols used to analyze the phytoplankton samples. The methods for enumerating and determining the viability of phytoplankton samples with the upgraded FlowCAM® had been previously developed by NRLKW using the analog version of the FlowCAM®.

The four groups and methods to be employed in support of the Phytoplankton Enumeration Workshop were:

- <u>**NRLKW**</u> – Manual microscopy using an epifluorescence microscope and Sedgwick Rafter slides. Cell viability determination via sample staining using the DNA stain SYTOX® Green (Invitrogen™, Carlsbad, CA; identifies dead cells).

- **WHOI** – Sample concentration by filtration combined with manual microscopy using an epifluorescence microscope to analyze the phytoplankton samples directly on the filter. Cell viability determination using a combination of vital stains (Cell Tracker™ Green CMFDA and Cell Tracker™ Blue CMAC to identify viable cells (Invitrogen, Carlsbad, CA)) and a DNA stain (SYTOX® Green) to identify non-viable cells.
- **MLML** – Flow cytometry and Pulsed Amplitude Modulated (PAM) Fluorometry at the BWTTF in Key West. With the flow cytometer, cell viability was determined using a combination of DNA stains (SYTOX® Green) and vital stains (Fluorescein Diacetate [FDA] and Calcein AM). MLML performed additional analyses, including ^{14}C determination of primary production, ATP and chlorophyll extractions, cell digestion assays and cell culturing, and most probable number (MPN) sample assessments. These measurements were primarily made on highly concentrated, nearly homogenous samples of either live or dead phytoplankton. These measurements were made at a combination of the BWTTF and at MLML (samples were shipped daily to MLML).
- **NRLKW/Fluid Imaging Technologies** – Updated Digital DSP based FlowCAM® and a new flow cell design. The DNA stain SYTOX® Green was used to identify non-viable cells.

The Phytoplankton Enumeration and Evaluation Experiment Workshop was conducted at the BWTTF at NRLKW from January 6 - 16, 2008. During the workshop, the teams independently evaluated seven separate phytoplankton samples with a variety of total concentrations and Dead:Live ratios. (Note that many other samples were evaluated early in the workshop as procedures were being developed and finalized.) The final sample analyzed (the 7th sample) was a more complex sample with test dust and dissolved organic and mineral matter.

The final section of the report provides conclusions that can be drawn from the phytoplankton enumeration experiment workshop and provides recommendations for additional work to further explore limitations of the methods that were evaluated.

2 EXPERIMENTAL PROCEDURES

The following sub-sections briefly describe the methods and procedures used during the workshop. More information and detailed procedures for each method are provided in Appendix B.

2.1 Phytoplankton Sample Preparation

Preparation of test samples at desired live and dead cell concentrations involved the serial dilution of live and dead *Tetraselmis* stocks (strain PLY 429) purchased by NRLKW from Reed Mariculture (Campbell, CA). To reduce the amount of microscope time required to prepare phytoplankton samples, it was decided to use Reed Mariculture's measurements of sample concentration provided with each purchased stock solution of live and dead *Tetraselmis*.

Prior to the start of the workshop, NRLKW demonstrated that using serial dilutions and the concentration values provided by Reed Mariculture allowed preparation of samples with total live or dead cell populations in the 10 cells mL^{-1} concentration range (within a factor of 2). This effort demonstrated the concentrations provided by Reed Mariculture were adequate for determining the dilutions required to produce the test samples of approximately known values for use by the workshop participants. The major advantage of this approach is that it required no additional microscope time, thus allowing NRLKW personnel to participate

in the workshop and analyze test samples using the methodologies previously employed during BWTE evaluations at NRLKW.

Initial work performed prior to the start of the workshop also showed the dead cells, which were killed by centrifugation, could be successfully stained using the DNA stain SYTOX® Green. At the start of the workshop, additional microscope observations of the phytoplankton cells from the dead stock using an epifluorescence microscope showed that the dead cells were autofluorescing in the green region of the spectrum on the outside of the cells. The signal from this fluorescence would initially mask the cell's chlorophyll *a* autofluorescence signal and would directly interfere with the SYTOX® Green fluorescence. Interestingly, as the cells were observed over a period of two minutes, it appeared that the illumination from the epifluorescence microscope broke down the compound on the outside of the cell that was autofluorescing. When breakdown occurred, the cells' chlorophyll *a* autofluorescence could be readily observed. It should be noted that only cells that were in the microscope's active field of view went through this transition.

Because the dead cells autofluoresced, a new method for preparing dead phytoplankton stock for the workshop was developed using ultraviolet irradiation. Dead, UV-treated cells did not autofluoresce and could be stained with SYTOX® Green to indicate cell viability. Unfortunately, an unanticipated outcome was the probability that variable rates of cell survival from the irradiation process impacted NRLKW's ability to prepare samples with accurate numbers of live and dead cells.

2.1.1 Sample Preparation Procedures

2.1.1.1 Basic Sample Preparation

Approximately 10 liters (L) of artificial seawater with a salinity of 30-31 practical salinity units (psu) was prepared for each of the first six experiments.

Dead *Tetraselmis* were prepared from the live *Tetraselmis* stock received from Reed Mariculture on a daily basis using a UV light. Live *Tetraselmis* stock was removed from an incubator and allowed to warm to room temperature (approximately 30 minutes). After the live stock was mixed by multiple inversions, three 20-milliliter (mL) aliquots of live, concentrated *Tetraselmis* stock were pipetted into individual plastic Petri dishes. The three Petri dishes were placed within approximately 13 cm of the UV light and remained under the UV lamp, uncovered, for 30 minutes. The Petri dishes were then removed from the lamp and allowed to sit on the lab bench for 60 minutes. Based on other workshop participants' experience in using UV to kill phytoplankton, it is believed that this provided sufficient time for the majority of the phytoplankton to die following the UV irradiation.

The stock of dead *Tetraselmis* was removed from the Petri dishes using a pipette and transferred to two 50-mL plastic containers prior to creation of the workshop test samples. Ten liters of artificial seawater was transferred to a 20-L Nalgene® container. Calculations based on the stock concentration values from Reed Mariculture were made to determine the volume of live and dead stock solution to be added to the 10 L volume to obtain the desired cell concentration levels and dead:live ratios. Prior to adding the stock solutions to the 10 L of artificial seawater, a volume of artificial seawater equal to the volume of the *Tetraselmis* stock to be added was removed from the 10 L sample. The algal stock solution was added to the artificial seawater, and the 10 L sample was mixed through a series of inversions.

From this 10-L sample aliquots were provided to each workshop participant. Typically, 1 L of test sample was provided to both the NRLKW and WHOI microscopy teams, 300 mL of test sample was provided to the NRLKW/Fluid Imaging Technologies team, and remaining sample volume (~ 7.5 L) was provided to the MLML team.

2.1.1.2 Total Suspended Solids/Total Organic Carbon (TSS/TOC) Preparation Method

The final (7th) phytoplankton sample provided to the teams was more complex than the other samples. To make this sample representative of samples processed during BWTE tests at NRLKW, mineral matter, dissolved organic carbon, and particulate organic matter were added to a low concentration of live *Tetraselmis* cells. The goal was to achieve a sample with the following characteristics:

- 20 live *Tetraselmis* cells mL^{-1}
- 5 mg L^{-1} Dissolved Organic Carbon (DOC)
- 5 mg L^{-1} Particulate Organic Matter (POM)
- 19 mg L^{-1} mineral matter (MM)

NRLKW had previously developed procedures to add decaffeinated iced tea, humic matter, and medium and coarse ISO test dust (Powder Technology Inc., Burnsville, MN) to obtain these concentrations for large-scale tests. Due to an error in calculation, however, the tea, humic material and test dust concentrations added to the artificial seawater were, in fact, 10 times higher than those required.

In order to prevent the clogging of the flow cells used in the FlowCAM® and MLML flow cytometer, it was decided to filter this sample stock using a 25 µm mesh screen to remove particles larger than 33 µm from the modified test sample prior to adding the live phytoplankton. Two liters were filtered using this process. Live *Tetraselmis* stock was then added to this solution with the goal of creating a test sample with a live cell concentration of approximately 20 cells mL^{-1}. Because only 2 L of this sample was generated, a smaller but still appropriate sample volume was provided to each of the workshop participants. Some additional sample was retained and provided to an outside lab which performed analysis of the TOC, POM and MM concentrations. It is important to note that the actual volume of the 7th sample analyzed by each of the workshop participants was the same or larger than that analyzed for the prior six workshop samples.

2.2 NRLKW - Microscopy

A 1-L workshop sample was provided to the NRLKW Microscopy group. After thorough mixing by a series of sample inversions, a 10-mL subsample was removed and dispensed into an 80 mL beaker. Using a sterile dropper, two drops of low-acidity food-grade vinegar (acetic acid) were added to the 10 mL sample to de-mobilize the living *Tetraselmis* cells. After the Tetraselmis sample and vinegar were well mixed by pouring the solution back and forth between the beaker and a second 80 mL beaker, a 1-mL subsample of the de-mobilized Tetraselmis sample was drawn from the 80 mL beaker using an Eppendorf micropipette and dispensed into a 1-mL centrifuge tube with 10 µL of 0.09 millimolar (mM) concentration SYTOX® Green. The centrifuge tube was mixed using a vortex mixer, and the tube plus phytoplankton sample and stain were placed in the dark to incubate for 10 minutes (min).

Following incubation, the prepared sample was again mixed using the vortex mixer, and a 1-mL sample was withdrawn and dispensed onto the Sedgwick Rafter slide. The Sedgwick Rafter slide is a specialized gridded (20 row x 50 column grid) microscopy slide for organism enumeration of a 1-mL volume sample. The cover glass was carefully placed onto the counting slide perpendicular to the long axis of the slide and

swung across the counting cell so it completely covered the sample. Careful alignment of the cover glass prevents air bubbles from being introduced into the sample and ensures that the slide contains a true 1-mL sample (Figure 1).

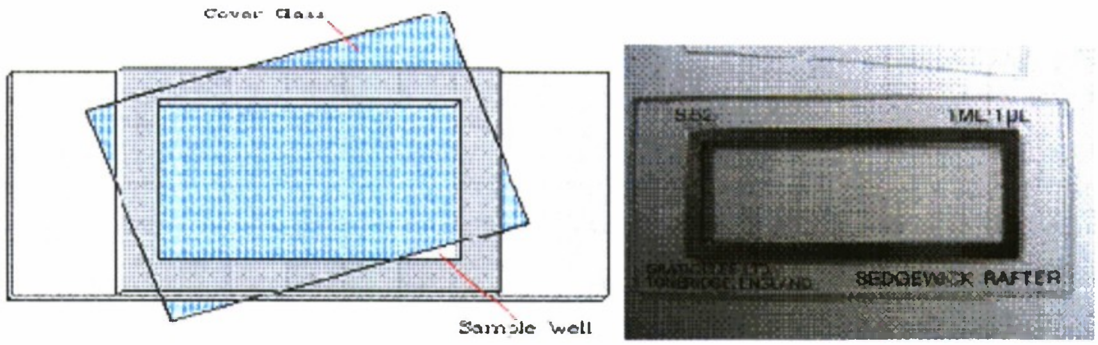

Figure 1. Sedgwick Rafter counting slide.

Once the sample was prepared on the Sedgwick Rafter slide, it was placed in the dark for an additional 20 min to allow phytoplankton cells to settle. When the stained *Tetraselmis* cells had settled, the Sedgwick Rafter slide was secured on the base of a Nikon E600 microscope with phase contrast and epifluorescence capabilities. The microscope was then used to identify and count both live and dead (as indicated by SYTOX® Green fluorescence) cells. SYTOX® Green is an impermeant fluorescent vital stain that binds to nucleic acids within cells with compromised membranes. Thus the SYTOX® Green fluorescence can be used to identify cells considered non-viable. By counting the total number of cells as well as those stained with SYTOX® Green, it is possible to determine, via subtraction, the live and dead concentrations.

A data tally sheet that was used to record sample analysis results is provided in Table 2. This sheet was specifically developed for supporting BWTE at NRLKW. Consequently, parameters such as "Sample Port", "Ambient Live" and "Ambient Dead" are not relevant to the analysis of workshop samples. The letters in the Table are randomly selected for each sample and correspond to specific rows on the Sedgwick Rafter slide. For each identified row on the data sheet, the number of live and dead cells were counted and recorded. Target live and dead cell concentration levels for each sample were entered into the "Sample Port" row during the workshop.

To determine live and dead concentrations, the total number of *Tetraselmis* cells were first counted on a given row of the slide. Once this count was completed, the microscope was switched to operate in the epifluorescence mode, and cells with SYTOX® Green staining of their nuclei (e.g., the dead cells) counted. The number of live cells in the row was then determined by subtracting the number of dead cells from the total number of cells in the row. This process was repeated for each of the 20 rows on the Sedgwick Rafter slide such that total live and dead cell counts mL^{-1} could be determined. For the majority of the workshop samples, cells in the entire 1-mL Sedgwick Rafter slide volume were counted.

Table 2. Sample data tally sheet used to record phytoplankton sample data. Note that this sheet was developed to support BWTE tests at NRLKW. As the "Sample Port" designation is not relevant to the analysis of workshop samples, the target sample live and dead cell concentrations for each sample were entered into this row. The columns marked Ambient Live" & "Ambient Dead" were not used in this workshop.

Phytoplankton Stained Live/Dead				
Date:				
Sample Port:				
Phytoplankton:				
Run:			Ambient	
	Live	Dead	Live	Dead
D				
B				
Q				
S				
K				
R				
H				
C				
L				
M				

2.2.1 Advantages & Disadvantages to Sedgwick Rafter Methodology

The advantage of manual microscopy using Sedgwick Rafter slides and SYTOX® Green vital stains is that the instrumentation required for this method is available in many laboratories. Further, sample incubation times are relatively short (30 minutes including settling times), and results can be generated fairly rapidly.

The major disadvantages of this method are that it takes several hours (4-5 hours) to characterize a sample (one sample comprised of 3 subsamples), and there is no archive of the observations produced. Additionally, the manual counts are subject to errors based on operator specific biases as well as from fatigue effects during times of extended microscope observations.

2.3 WHOI – Microscopy

2.3.1 Methods

The WHOI group also used microscopy and stains for sample analysis, but the subsample volume analyzed was significantly greater than could be accomplished using the 1-mL Sedgwick Rafter slides. Three subsamples (usually 5 mL) were prepared from each 1-L workshop sample. The subsamples were pipetted into separate 15-mL disposable centrifuge tubes and vital stains added. Cell Tracker™ Green CMFDA (5-chloromethylfluorescein diacetate) and Cell Tracker™ Blue CMAC (7-amino-4-chloromethylcoumarin), and SYTOX® Green stains were used. (See Appendix B for stain concentrations.) The subsamples and stain were mixed by inversion and incubated in the dark at room temperature for 45 minutes. After incubation 100% formalin was added to kill and preserve sample organisms.

The subsamples were then filtered onto separate 5µm 25mm Whatman Cyclopore polycarbonate track-etched membrane filters and rinsed three times. Each filter was placed onto a glass slide, 25 µL of glycerin was added, and a cover glass applied. Slides were then stored and refrigerated in the dark for up to four days until examined with a Lietz Diaplan microscope (equipped with a 100-watt high pressure mercury light and a Chroma filter set) at 200X magnification. For low density concentrations, the entire filter was enumerated. For high density samples, two or more sweeps were made until 400 organisms were counted.

Full details of this method are found in Appendix B.

2.3.2 Method Advantages/Disadvantages

This method affords the user several key advantages in comparison to the other methodologies utilized during this workshop. These include the ability to easily process (label) different volumes of samples; flexibility in sample enumeration; unambiguous detection of live and dead cells; chlorophyll *a* signal left intact to aid in the enumeration of dead cells; low cost of materials; simple laboratory protocols; processed sample stability; ability to microscopically inspect the sample population following sample processing; and the possibility of simultaneously dual labeling a sample with live and dead vital stains.

Some disadvantages to this method are: labeling variability with live vital stains; moderate daily sample throughput; use of hazardous chemicals and the cost of vital stains if large sample volumes are processed.

The microscope is a major expense, and thus, a disadvantage associated with this method. Most of the other required equipment needs are low in cost and are usually found in most biological laboratories.

2.3.3 Additional Information and Recommendations for Method

As mentioned above, various sample amounts ranging from µL to L volumes can be labeled with live and dead cell vital stains so long as the proper final concentration of stain can be achieved (5µM Cell Tracker™ Green CMFDA and Cell Tracker™ Blue CMAC, or 0.5 µM SYTOX® Green). The volume of sample that should be processed is primarily driven by the expected cell densities and the required measurement accuracy (which increases with the number of cells counted). Other factors, however, such as sample biomass and detrital matter concentrations are also important in determining the specific sample volume to process. Given these factors, a quick microscopic evaluation of an unfiltered sample prior to processing can be used to help define the volume of a particular sample to process.

Once processed, the sample filter can be counted in its entirety or counted partially depending on the selected target cell densities. Andersen and Throndsen (2003) provide a good summary of how to count slides with various sample densities and provide a statistical table documenting the percent error associated with different cell counts. A high-quality microscope is required to quickly, comfortably, and reliably obtain an accurate cell count. Key microscope features to consider are a 100-watt high-pressure mercury light source, tailored optics for fluorescence microscopy, and fine-tuned fluorescence filter sets with long-pass emission filters that allow uninhibited visualization of the live and dead cell fluorochromes in addition to fluorescently active cell pigments. A good-quality, color, digital camera to document experimental results is also suggested.

Long-pass fluorescence emission filter sets are superior to band-pass emission filters in several aspects: they often allow a brighter level of signal to be visualized, they provide a more realistic color of the vital stain fluorescent product, and they allow for all cellular fluorescent colors (e.g. Cell Tracker™ Blue CMAC (blue

fluoresence), SYTOX® Green (green fluoresence) and chlorophyll *a* autofluorescence (red fluoresence)) above their cutoff point to be seen simultaneously. The ability to observe these fluorescent signals simultaneously in phytoplankton cells is extremely beneficial when enumerating and classifying the viability samples. Pigment fluorescence in algal cells is usually red (chlorophyll *a*), or orange (phycoerytherin), and this fluorescence can be masked to various levels by live vital stains such as Cell Tracker™ Green CMFDA and Cell Tracker™ Blue CMAC. The pigment fluorescence signals are typically sufficiently strong and easily observed in freshly killed cells that are labeled with SYTOX® Green due to the small size of the nucleus (where SYTOX® Green fluorescence occurs) compared to the cytoplasmic contents of most phytoplankton cells (where pigment fluorescence occurs). In this case, the pigment fluorescence allows for quick recognition of phytoplankton cells with the highly localized and intense SYTOX® Green fluorescence allowing for a rapid evaluation cell viability. Pigment fluorescence can also be used to identify dead cells in a sample labeled with only a live cell vital stain if a companion dead cell stain was not utilized. In this situation, the live cell would be illuminated with the vital stain in addition to some residual pigment fluorescence (if not fully masked), whereas the dead cell would only have pigment fluorescence.

The sample protocols as outlined in the methods sections are simple to follow and do not require a high level of laboratory expertise. Unfortunately, sample throughput is low with this protocol. Comfortably, 6 samples can be labeled and enumerated in 6 hours, but this quantity is dependent on the number of target organisms being enumerated, the complexity of the sample in terms of biodiversity, and sample matrix factors. A breakdown of the time requirements for this method are as follows: Two hours are required to label 6 samples whether they be 2 separate samples with 3 subsamples each, or 1 sample being labeled with 2 vital stains with three subsamples each, or some other combination ultimately resulting in 6 processed samples. The labeling consists of preparation of the reagents, a 45-minute sample incubation time, preservation and filtration of the samples, mounting the filters on slides and cleanup. The remaining 4 hours is for sample enumeration, including allowances for data entry.

Labeled samples are stable for at least 3 days, and perhaps longer, provided that the samples are kept at 4 °C and dark. This provides the potential for analyzing more sub-samples as measurements can be conducted over several days versus several hours. Additional studies should be conducted if long-term sample stability is deemed to be a desired criterion.

It is believed by the authors that samples can be simultaneously dually labeled with live and dead cell stains as long as the reporter fluorochromes are spectrally separated from one another, e.g., using Cell Tracker™ Blue CMAC and SYTOX® Green. This experiment was attempted using SYTOX® Green and Cell Tracker™ Green CMFDA, but the reporter fluorochromes in the two vital stains are too similar in color. It became difficult to distinguish which vital stain was reacting within a specific cell in many instances because of the variable labeling of Cell Tracker™ Green CMFDA in live cells. This variability is believed to be due to the physiological status of the cell. A cell that is very healthy will exhibit robust Cell Tracker™ Green CMFDA labeling, while a cell that is alive, but metabolically inactive, will have a lower degree of Cell Tracker™ Green CMFDA label visible in the cell. This low level Cell Tracker™ Green CMFDA labeling could be confused with a SYTOX® labeling if both vital stains were simultaneously added to the sample. The lack of a long pass emission filter on the UV filter set housed in the Lietz microscope precluded testing of the Cell Tracker™ Blue CMAC and SYTOX® Green combination. These two reporter fluorochromes are spectrally separated, and one would expect this combination of live and dead vital stains would be compatible, and distinguishable, in the same sample.

One concern in using vital stains on future samples collected from ballast test trials is the resulting status, or condition, of the cells following treatment. In the preliminary trials during this workshop, it was found that dead *Tetraselmis* cells purchased from Reed Mariculture had an inherent broad-spectrum autofluorescence that precluded the use of vital stains as it was impossible to determine if any dead or live stain was taken up within the cells. Upon realizing this problem, it was decided to use UV to irradiate live cells to kill them. These irradiated cells did not produce this broad-spectrum autofluorescence but rather had the expected chlorophyll *a* autofluorescence associated with freshly killed cells and allowed unambiguous stain labeling. Whether or not different ballast water treatment technologies will produce dead phytoplankton cells that have a broad-spectrum fluorescence is unknown and may be a function of a particular treatment type. As such, one must proceed with caution if vital stains are to be used to assess live and dead phytoplankton within these samples. To help account for broad-spectrum auto-fluorescence in algal cells, an unlabeled control should be prepared and examined using the filter sets that will be employed for vital stain analysis.

2.4 Moss Landing Marine Laboratory – Various Methods

This portion of the report was compiled by the NRLKW staff from several materials provided by MLML. All available information on methodology used for workshop samples and high density samples is presented in this section.

2.4.1 Flow Cytometry

A step-by-step protocol of the procedures used for MLML's flow cytometry work was not provided to NRLKW. It is known that MLML researchers used a Becton-Dickinson FACSort flow cytometer operated with CellQuest Acquisition Software and a CYTOWIN 4.31 analysis software package.

The settings used with the CYTOWIN 4.31 analysis software package were FSC: E-1, SSC 275, FL1 350, FL2 350, FL3 275 threshold FL3 100 with all voltages set in log mode. It is believed that these settings were used by MLML to classify detected particles as living cells, dead cells, or debris based on two fluorescence measurements and scatter measurements. With this information alone, it is impossible to assess the efficacy of these settings to distinguish the various phytoplankton classes. The settings are, however, only relevant to the specific flow cytometer, acquisition software and analysis software that was used by MLML to support the workshop.

The instrument was operated with a flow rate of 60 µL min^{-1} with data generally collected over 15 minutes per sample (some runs were shorter as a result of power failures). This implies that approximately 0.9 mL of sample volume were characterized by the MLML Flow Cytometer. In general, the group attempted to analyze 5 subsamples of both stained and unstained samples.

Stains were used at the following concentration levels by MLML:

- SYTOX® Green – 0.5 µM – incubation time 15 min in the dark – approximately 30 – 40 min to stain and process a subsample
- Calcein AM – 10 µM- incubation time 30 min in the dark – estimate 45- 60 min to stain and process a subsample.
- FDA – 10 µM- incubation time 30 min in the dark – estimate 45- 60 min to stain and process a subsample.

Phytoplankton Enumeration and Evaluation Experiments

A review of the MLML flow cytometer data shows how the flow cytometer can be used in conjunction with vital stains (in this case SYTOX® Green) to distinguish both live and dead cells (Figure 2). Again, it is unclear how the settings provided above are used to interpret these data and perform classification or how refinements in the software analysis parameters might impact classification results.

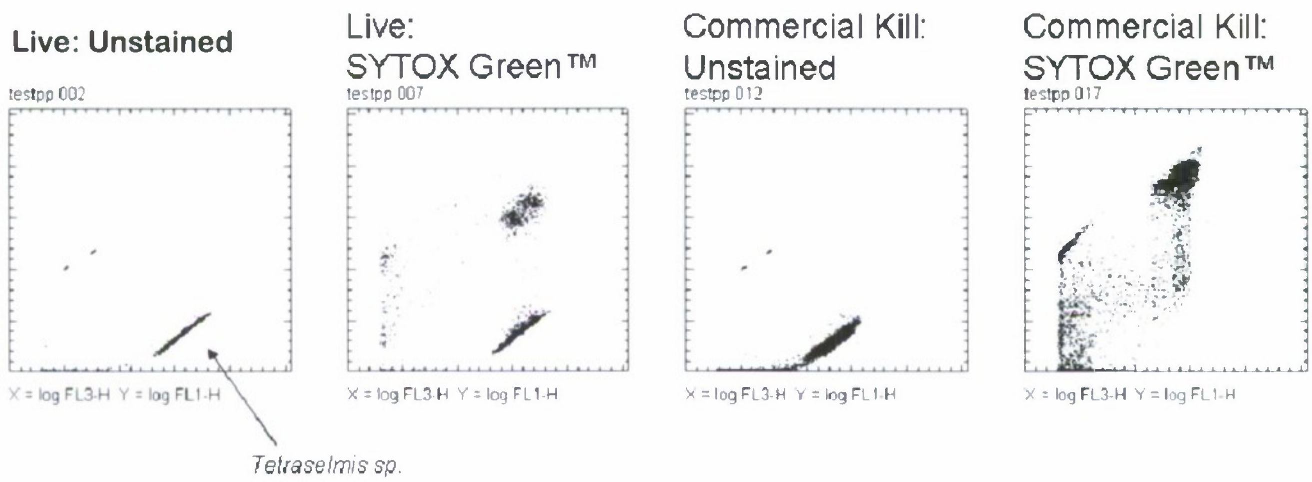

Figure 2. Raw flow cytometry data from MLML.

Additional raw data from the MLML Flow Cytometer, shown in Figure 3, were collected from the last, complex workshop sample that included suspended and dissolved solids. A review of the data indicates that because SYTOX® Green also stained the test dust particles, dead cells could not be easily identified using the flow cytometer. The dead cells' fluorescence signals overlap with test dust fluorescence signals (Figure 3). The data also show when the samples are stained using FDA, the flow cytometer can be used to both identify the dead and live phytoplankton cells. Again, it is unclear how the analysis software settings are used to interpret these data and perform classification or how refinements in the software analysis settings might impact classification results.

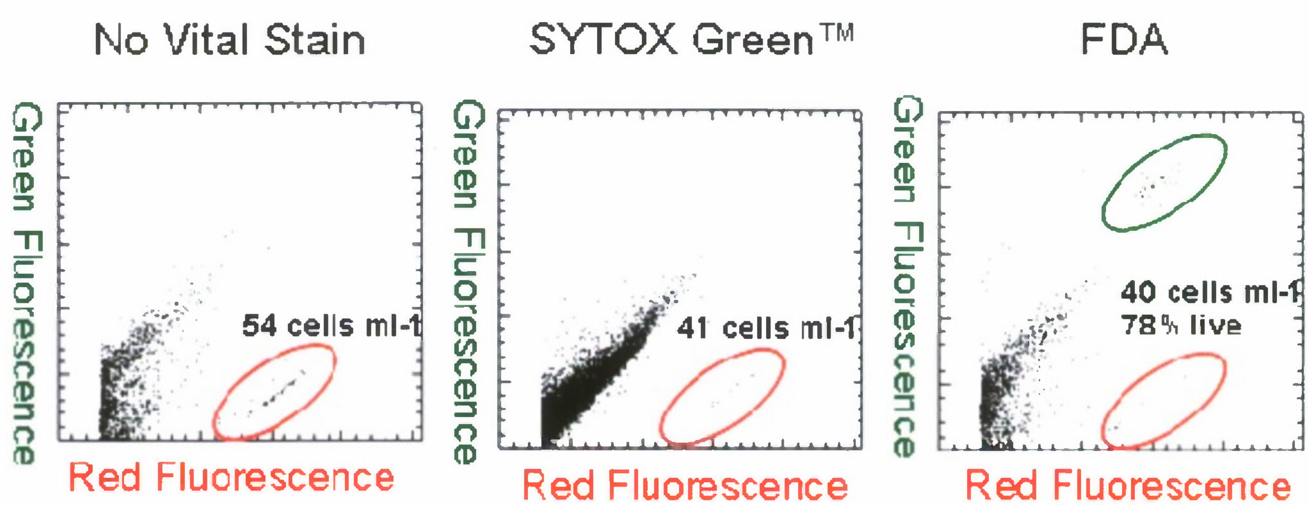

Figure 3. Flow cytometry data from MLML showing improved live/dead detection with FDA in a workshop sample with added test dust.

2.4.2 Pulsed Amplitude Modulated (PAM) Fluorometry

This technique was used to evaluate the physiological condition of photoautotrophic organisms using a Walz Water-PAM fluorometer (Heinz Walz GmbH , Effeltrich, Germany). The standard Walz 3 mL optical cuvet chamber was used only for samples of high cell density; growth media was used to dilute samples that were highly concentrated. This unit was not used with the workshop samples.

The workshop samples were evaluated using a Walz flow-through 350 mL cuvet system without dilution; the flow through system offers a ten-fold increase in sensitivity over the fixed cuvet configuration. The samples were kept in the dark until they were introduced in the cuvet systems under dim light, held for 3 minutes in the darkened cuvet, and analyzed with the PAM light-curve protocol. Values for the dark adapted determination of F_v/F_m are reported for many of the workshop samples.

2.4.3 Most Probable Number (MPN) Determination of Viable Cell Concentration

The MPN technique for determining viable cell densities is a culture-based method that relies on growth of target microorganisms monitored after quantitative serial dilution to extinction (that is, diluted to such a degree that no target cells are expected to grow in the most dilute series of subsamples). In concept, the technique can be applied to phytoplankton if suitably long incubations are employed to account for the slower growth rates of phytoplankton (about one doubling per day). We used whole cell fluorometric analysis on a sensitive SPEX Fluoromax 2 fluorometer to determine growth in fluid MPN cultures (3 mL) incubated under continuous light and controlled temperature. Aliquots (3 mL) of undiluted samples were placed in each of 5 clear glass culture tubes. Each tube was then diluted serially five times (ten-fold dilution for each series) with fresh f/4 seawater media yielding a suite of 30 tubes, each filled to 3 mL (5 subsamples x 6 dilutions; relative dilutions were 1 through 1×10^{-5}, in log increments). All culture tubes were identical and could be used as sample cuvets introduced directly in the fluorometer for fluorescence readings at excitation and emission wavelengths of 430 nm and 680 nm, respectively. MPN sample series were monitored every three days for three weeks to ascertain growth as indicated by fluorescence readings that were two-fold higher than the blank (filtered Barnstead NANOpure® water). The final MPN estimate of live cells mL^{-1} were obtained through computerized MPN tables for five subsamples.

MPN results were generated for three workshop samples and are presented in section 3.4.

2.4.4 Additional Measurement Methods

The following methods were also proposed by MLML for analyzing workshop samples. However, these methods were only applied by MLML to analyze highly concentrated samples of mostly live or dead cells that were produced by NRLKW for MLML. Although the samples differed from those of the workshop, the methods are provided for completeness and because they are still potentially relevant to determining the concentration and viability of phytoplankton samples. Results of these methods are not provided.

2.4.4.1 Photosynthetic rates: Carbon 14 Uptake (^{14}C) uptake

The ^{14}C technique provides a sensitive and simple means to evaluate bulk photosynthetic rates from the total phytoplankton community in any sample. A standardized incubation procedure was setup at MLML, California, where radioisotope facilities were available. Samples were shipped overnight from NRLKW to MLML for this purpose. Three shipments were received, each containing 'live' and 'dead' samples produced from two independent preparations made during the morning and afternoon preparation times at NRLKW. Two pairs of 'live/dead' samples were included in each shipment.

Samples, shipped with ice packs, were processed immediately upon arrival at MLML facilities. All ^{14}C incubations were made in triplicate, using 15-mL polypropylene centrifuge tubes as incubation vessels. Quantitative sample volumes (1-4 mL) were pipetted into the incubation vessels, filled to 14 mL with f/4 seawater media (to ensure nutrient saturation) and inoculated with quantitative additions of aqueous ^{14}C-bicarbonate solution, nominally 0.3 µCi (micro-curie). The samples were placed on a rotating wheel exposed to 30 $\mu E\ m^{-2}\ s^{-1}$ irradiance from two fluorescent lamps; the incubation was made for 24 hours under continuous illumination in a 14 °C walk-in cold room. The samples were terminated by filtering the entire content of each vessel onto 25 mm GF/F filters. The filters were then fumed over HCl for 3 min to remove contaminate inorganic ^{14}C and were immersed immediately in scintillation vials containing 4-mL ScintiSafe* (Fisher Scientific, Pittsburgh PA) cocktail. Samples were counted on a Packard 2200L scintillation counter. The mean value of three incubation tubes filled with 14 mL f/4 media served as a blank that was subtracted from each subsample to compute final counts. A 50 µL aliquot from each blank tube was withdrawn prior to filtration and added directly to scintillation vials containing 4 mL NaOH-buffered scintillation cocktail to compute the total ^{14}C added to each sample. Specific activity (counts per minute per µgC) was computed assuming a total inorganic seawater concentration of 25 mgC L^{-1}.

The ^{14}C uptake measurements were not made on any of the mixed workshop samples. These measurements were made on highly concentrated samples of mostly dead or live phytoplankton. As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

2.4.4.2 Chlorophyll a determination

Chlorophyll *a* was determined fluorometrically using single-step extraction technique. Samples were extracted in 90% acetone overnight at -4 °C; the extract was vortexed and diluted quantitatively with 90% acetone for readings taken on a Turner TD700 fluorometer fit with single-step, optical interference filters. The fluorometer was calibrated with spectrophotometrically quantified pure chlorophyll *a* standards in 90% acetone.

As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

2.4.4.3 Adenosine triphosphate (ATP)

Measurements of particulate ATP were made using luciferin-luciferase based photometric assay (Karl 1980). Samples were harvested on 25 mm GF/F filters, extracted immediately in 4 mL boiling buffer (20 mM) and frozen until analysis on a Turner 20/20 ATP photometer. Reagents and ATP standards were supplied in a Promega Enlighten ATP assay kit.

As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

2.4.4.4 Method Advantages/Disadvantages

Even though the results are interesting, because of the lack of detailed information on the methods used on the workshop samples, it is not possible to list the advantages and disadvantages of the MLML methods.

Incomplete references provided with MLML methods include: (Welschmeyer et al. 1991) for ^{14}C techniques and (Welschmeyer 1994) for ATP measurements.

2.5 NRLKW/Fluid Imaging Technologies –FlowCAM®

The FlowCAM® system is a commercially available system that images particles and provides data for each particle passing the sensor. A brief description of the instrument and those modifications to the initial design requested by NRLKW is provided.

2.5.1 FlowCAM® System

NRLKW has been exploring the use of Fluid Imaging Technologies' FlowCAM® system (Figure 4) as a means for automating the analysis of phytoplankton samples since 2004. Since that time, NRLKW has worked in conjunction with Fluid Imaging Technologies to implement the changes required in the FlowCAM® system to allow this instrument to be used for the automated analysis of phytoplankton samples from the BWTTF.

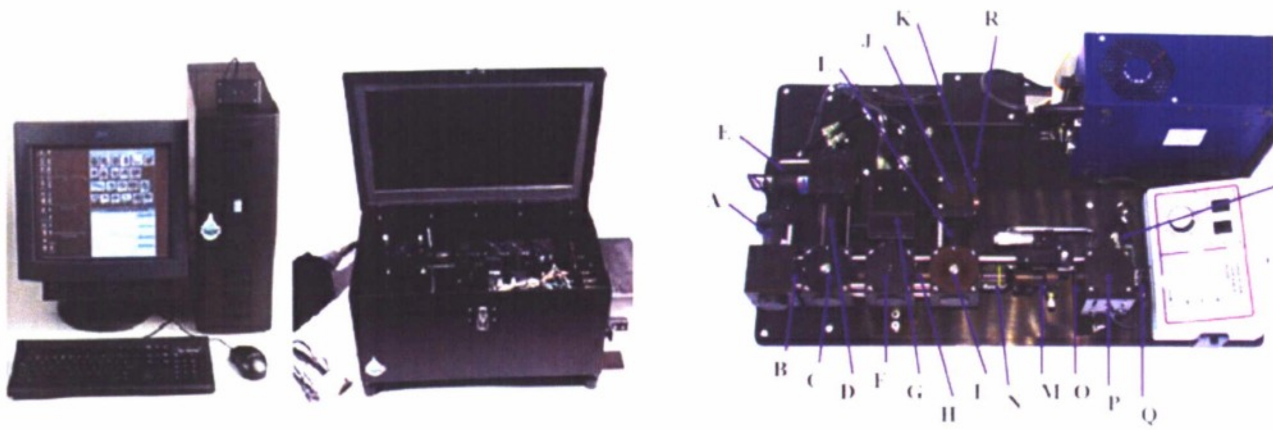

Figure 4. FlowCAM® components and layout.

The FlowCAM® System and its components, depicted in the two photographs on the left of Figure 4, consist of a personal computer (PC) used to control the system and to archive the collected data and a separately housed opto/mechanical instrument. The instrument itself is shown in the photograph on the right in Figure 4. Housed in the instrument are all the mechanical components required to bring the samples through a flow cell (pumps, controls, flow cell holder, etc.). Also in the instrument are the optical components used to induce and measure florescence from particles passing through the flow cell as well as a detector that monitors particle-induced forward scatter from this same laser. When a particle is detected by either of the florescence channels or by the forward scatter channel, it is imaged as it passes through the flow cell. Imaging is accomplished using a unique optical configuration that uses a "flash" LED (light emitting diode) to illuminate the particle and a unique microscope objective/camera/custom optical element configuration that extends the microscope objective's depth of focus. As a result, this system produces very high-quality images of detected particles.

During the course of working with the FlowCAM®, NRLKW has requested a number of modifications of the instrument that was initially purchased in 2004. These modifications include:

- Upgrade to a four-channel system (required for the simultaneous measurements of fluorescence at two wavelengths and forward scatter)
- Upgrade to a high resolution progressive video camera (improves the resolution of detected particles)

- Upgrade to a blue laser (required for compatibility with both DNA staining and metabolic dyes for viability assessment)
- Improve the sensitivity of the forward scatter channel (required as a result of the shift to a blue laser and a desire to have measurable forward scatter signals for phytoplankton sized particles).

It should also be noted that many of these modifications have subsequently been adopted in commercially available Fluid Imaging Technologies Systems.

The FlowCAM® produces a high-resolution image of each detected particle as well as useful florescence intensity signals (the first at the chlorophyll *a* autoflorescence wavelength and the second at a wavelength optimized for compatibility with the SYTOX® DNA staining dye) and a forward scatter intensity signal. The florescence and scatter intensity levels, in conjunction with image features generated by the FlowCAM® and additional features extracted from the high resolution imagery using in-house developed algorithms, offer the potential to fully automate the classification and enumeration of surrogate and indigenous phytoplankton and to determine their viability.

However, work that NRLKW performed on the FlowCAM® in 2007 identified a serious problem with the instrument that would preclude its use for quantifying phytoplankton concentrations and viabilities in BWTTF samples [Nelson, Riley, Hogan, Lemieux (2007)]: when the two fluorescence channels and the forward scatter channels were operated simultaneously, all of the channel outputs became corrupted. The signal corruption was so great that dead cells would be classified as living ones based on the apparent lack of a SYTOX® fluorescence signal. The corruption also prevented the unit triggering on a forward scatter level appropriate for the reliable detection of phytoplankton.

NRLKW made Fluid Imaging Technologies aware of the issues with the NRLKW FlowCAM® unit. In turn, Fluid Imaging Technologies was able to verify that the problems identified with the FlowCAM® were endemic to all of the "Analog" versions of this system that had been produced up to that time (approximately 80 units). Fortunately, Fluid Imaging Technologies had been working on the development of a Digital Signal Processor (DSP) based version of the FlowCAM®, which uses a DSP instead of analog circuitry to generate the fluorescent and scatter channel output signals. Initial experiments with the DSP-based FlowCAM® showed this technical approach obviated the problem inherent to the "Analog" version of the FlowCAM®.

Fluid Imaging Technologies was working on one additional change in the FlowCAM® that was of interest to NRLKW, i.e., the design of a new type of flow cell to allow the entire width of the flow cell to be imaged. The original flow cells allowed approximately 70 – 80 % of this width to be imaged. This restriction could create significant difficulties with the low cell concentrations that were anticipated in the Phytoplankton Enumeration and Evaluation Workshop sample matrix (as measurements are simply scaled to a higher value based on the flow cell's non-imaged area). The new flow cells were manufactured in a glass substrate that allowed their widths to be significantly reduced from the "free standing" flow cells that had been previously used with this system. At the time of the Workshop, prototypes of the new flow cell designs were available only in a size compatible with the 10X optical arrangement of the FlowCAM®. Most of NRLKW's previous work with phytoplankton had been done with the 20X optical arrangement of the FlowCAM®; however, the potential for missing phytoplankton cell as a result of the un-imaged areas of the original flow cell design presents a greater concern than the loss of spatial resolution that results from decreasing the FlowCAM®'s magnification from 20X to 10X. A new flow cell design that is compatible with the 20X optical configuration has become available from Fluid Imaging Technologies since the phytoplankton workshop.

It was desired to upgrade the NRLKW FlowCAM® unit prior to the start of the phytoplankton workshop. The NRLKW FlowCAM® had been modified multiple times in the past, and its configuration was not necessarily fully representative of the current systems being produced by Fluid Imaging Technologies. Further, the "analog" FlowCAM® could not be used to reliably determine if detected phytoplankton were viable, nor could it be known that the unit was triggering reliably on detected phytoplankton cells (as a result of issues with the forward scatter intensity measurements). Lastly, the lack of complete flow cell coverage with the FlowCAM®'s imaging system was a concern with the low concentration samples that were to be the emphasis of planned workshop measurements.

In November 2007, NRLKW and Fluid Imaging Technologies agreed to participate together in the phytoplankton enumeration experiments. As part of their participation in the workshop, Fluid Imaging agreed to update the NRLKW FlowCAM®. Specifically, the unit was first made optically (fully) and mechanically (mostly) identical to their current production units. The unit was upgraded to the DSP version, and the flow cell holder was modified to accommodate the new flow cells that were under development by Fluid Imaging Technologies. Fluid Imaging Technologies also agreed to fully train NRLKW to use the updated FlowCAM® and to perform initial experiments to demonstrate that the new version of the FlowCAM® overcame previous difficulties. Lastly, Fluid Imaging agreed to provide support for the first several days of testing associated with the Phytoplankton Enumeration Workshop. Researchers at NRLKW developed methods for determining cell viability using the FlowCAM® to be utilized during the workshop. Additionally, NRLKW personnel operated the FlowCAM® through all the measurements that were made during the phytoplankton enumeration experiment workshop. Through this arrangement, the NRLKW/Fluid Imaging Technologies team was able to make the current state of the art FlowCAM® available at the workshop.

It is important to note that the FlowCAM® was the only technique that was critically evaluated as part of this workshop. It is likely that an in-depth analysis and evaluation of any of the other workshop methods would indicate similar issues as those that are described in the following report sections.

2.5.2 FlowCAM® Methods

The following methods were used to process all but one of the Phytoplankton Enumeration Workshop samples. This method had to be modified to process the last workshop test sample because if its complexity (the addition of test dust, dissolved solids, etc).

As with the manual microscopy methods, samples for FlowCAM® analysis were stained with SYTOX® Green. SYTOX® Green itself autofluoresces and thus creates a background signal, which significantly reduced the reliability and sensitivity of FlowCAM®'s photomultiplier tubes. Figure 5 shows a SYTOX® Green blank created by adding 10 µL of 5 mM SYTOX® Green to 1 mL of artificial seawater. The flow cell's entire width is visible in Figure 5, and the fluid's autofluorescence is visible in the red ellipse.

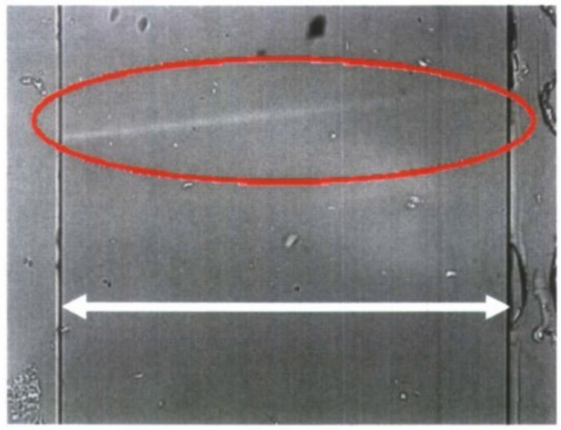

Figure 5. SYTOX® Auto fluorescence in flow cell. with 10 µL of 5 mM SYTOX® added to 1 mL of artificial seawater.

It was learned from other workshop participants that the concentration of SYTOX® Green could be significantly reduced without reducing the ability to detect dead cells. As can be seen in Figure 6, reducing the SYTOX® Green concentration to 10 µL of 0.09 mM SYTOX® Green in 1 mL of artificial seawater largely eliminated the autofluorescence signal (Figure 6). This is the concentration that was used throughout the workshop for the FlowCAM® experiments. Further exploration of the efficacy of SYTOX® Green staining capability over a range of concentrations is recommended to optimize the concentration for detecting non-viable phytoplankton in solution.

A SYTOX® Green blank was generated and its photograph collected at the beginning of each run. Once the stain blank was generated, workshop samples were analyzed in the following manner. The pump on FlowCAM® was adjusted such that only one particle (cell, debris, etc) was in the instrument's field of view at one time. A 1-mL SYTOX® Green blank was pumped through the flow cell to pre-condition FlowCAM®. A 1-mL SYTOX® Green stained phytoplankton sample was then introduced. When the sample reached the neck of the funnel used to bring the sample into the FlowCAM®, a 0.5-mL SYTOX® Green blank was introduced to ensure all phytoplankton cells were pumped through the flow cell. This was followed by a second 0.5-mL blank. It took approximately 30 minutes to completely process a 1-mL phytoplankton sample.

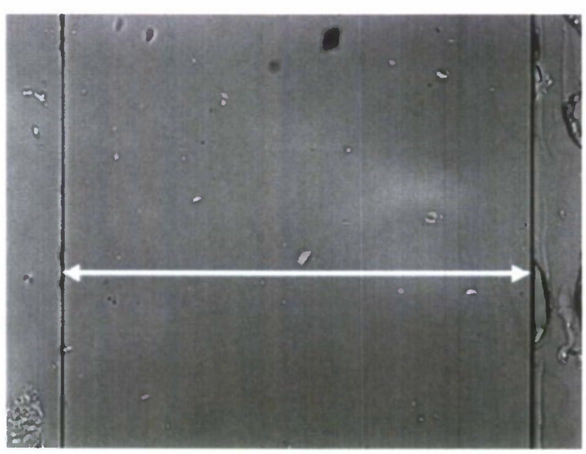

Figure 6. SYTOX® Auto fluorescence is not observed in the flow cell when 10 µL of 0.09 mM SYTOX® is added to 1 mL of artificial seawater.

Processing of the phytoplankton images and data was standardized into a multi-step process. The data were first imported into Fluid Imaging Technologies' Virtual Spreadsheet software which displayed four data plots as well as data on detected particles (Figure 7). The plot on the upper left shows chlorophyll *a* fluorescent intensity (CH 1 Peak) as a function of equivalent sphere diameter (ESD). The ESD comes from image process operations rather than forward scatter as is the case with standard flow cells. The upper right plot is SYTOX® Green fluorescence intensity (CH 2 Peak) against ESD. The lower left is forward scatter intensity (CH 3 Peak) against ESD. The lower right is chlorophyll *a* fluorescence intensity as a function of SYTOX® Green fluorescence intensity (CH 1 against CH 2). This plot (lower right) was used to process the phytoplankton data in the Virtual Spreadsheet. Information about the detected particles shown at the bottom of Figure 7 includes the number of particles detected.

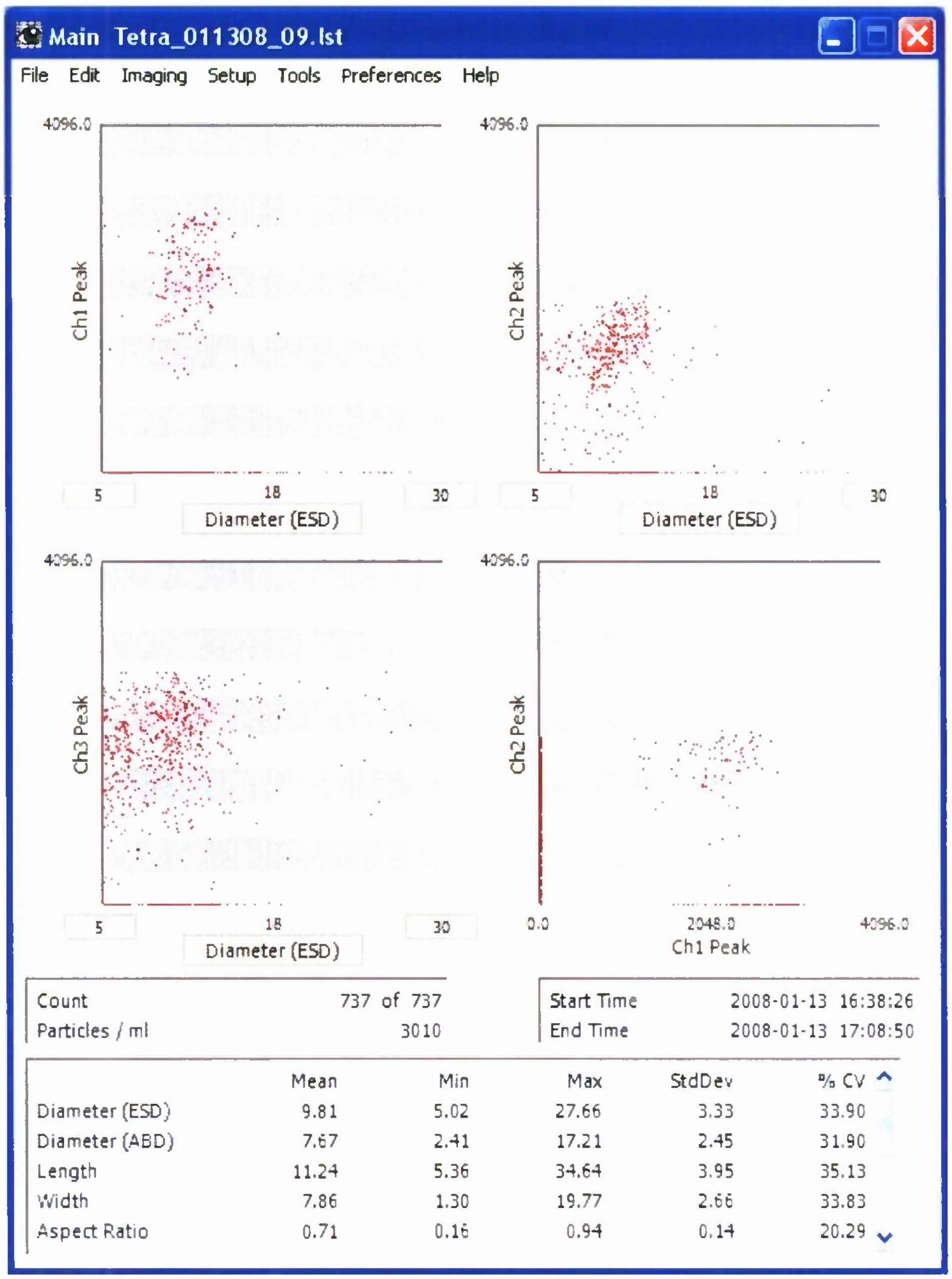

Figure 7. FlowCAM® GUI display for a workshop sample.

The first step to refine the data was to identify particles with chlorophyll *a* but no SYTOX® Green fluorescence (i.e., live cells). Using the lower right plot and Virtual Spreadsheet, the operator used the mouse to encircle particles along the CH1 axis that met this condition. The black ellipse in Figure 8 indicates these particles, and the display of the graphical user interface (GUI) shows the 95 particles selected by the operator from the 737 total particles. The operator then reviewed the individual particles to select any particle believed not to be phytoplankton. (Note the 3 images outlined in red.) These particles were detected in the same field of view as phytoplankton cells and were assigned the same values by FlowCAM®.

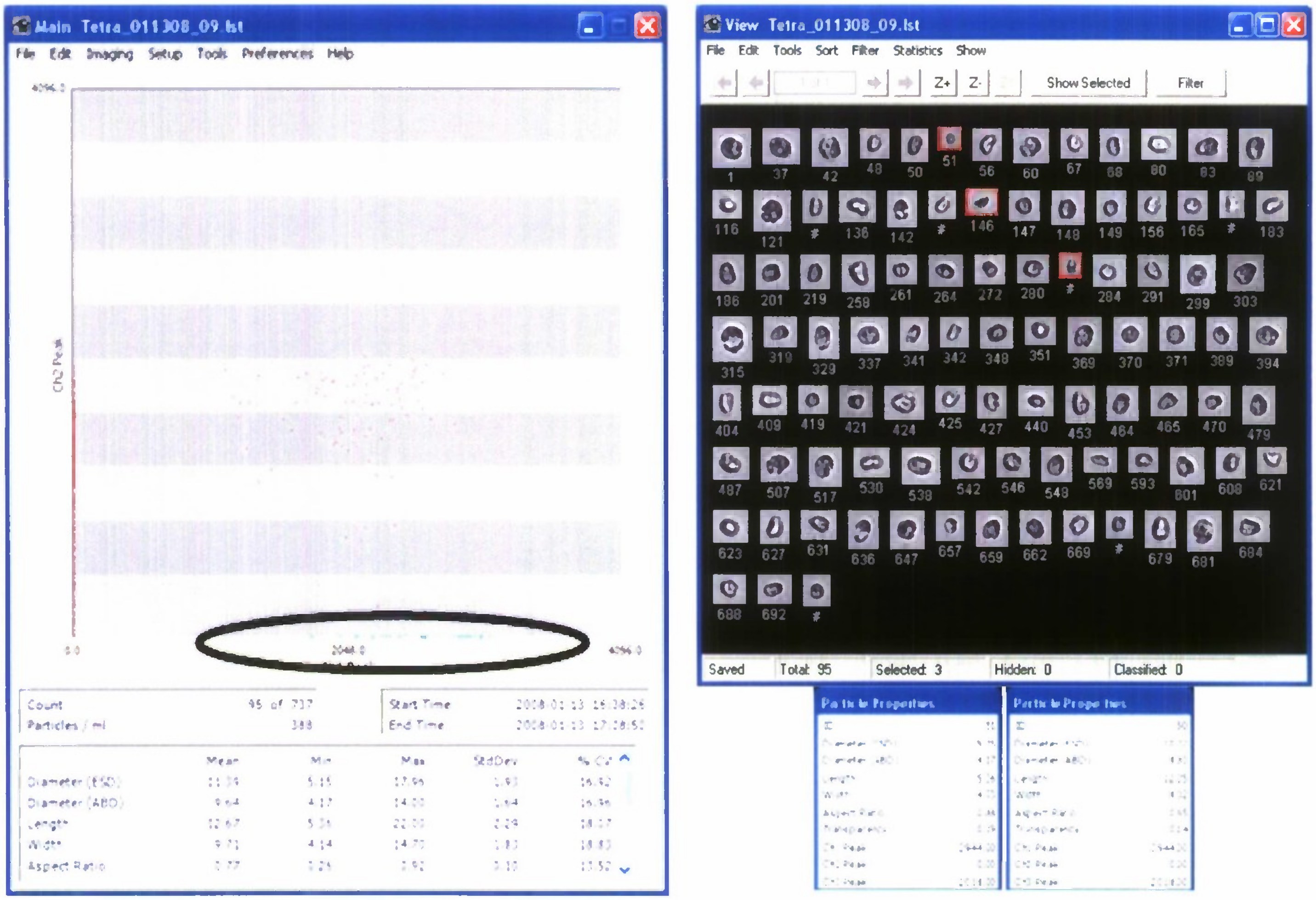

Figure 8. Particles selected had chlorophyll *a* fluorescence (autofluorescence) but no SYTOX® fluorescence.

Particles with both chlorophyll *a* and SYTOX® Green were next selected as shown in Figure 9. These were classified as non-viable cells. Upon review of the 83 particles thus selected, the operator searched for those thought not to be phytoplankton. Again, these particles were in the same field of view as phytoplankton cells and were assigned the same values. In this instance 80 of the 83 particles detected were uniquely identified as non-viable phytoplankton.

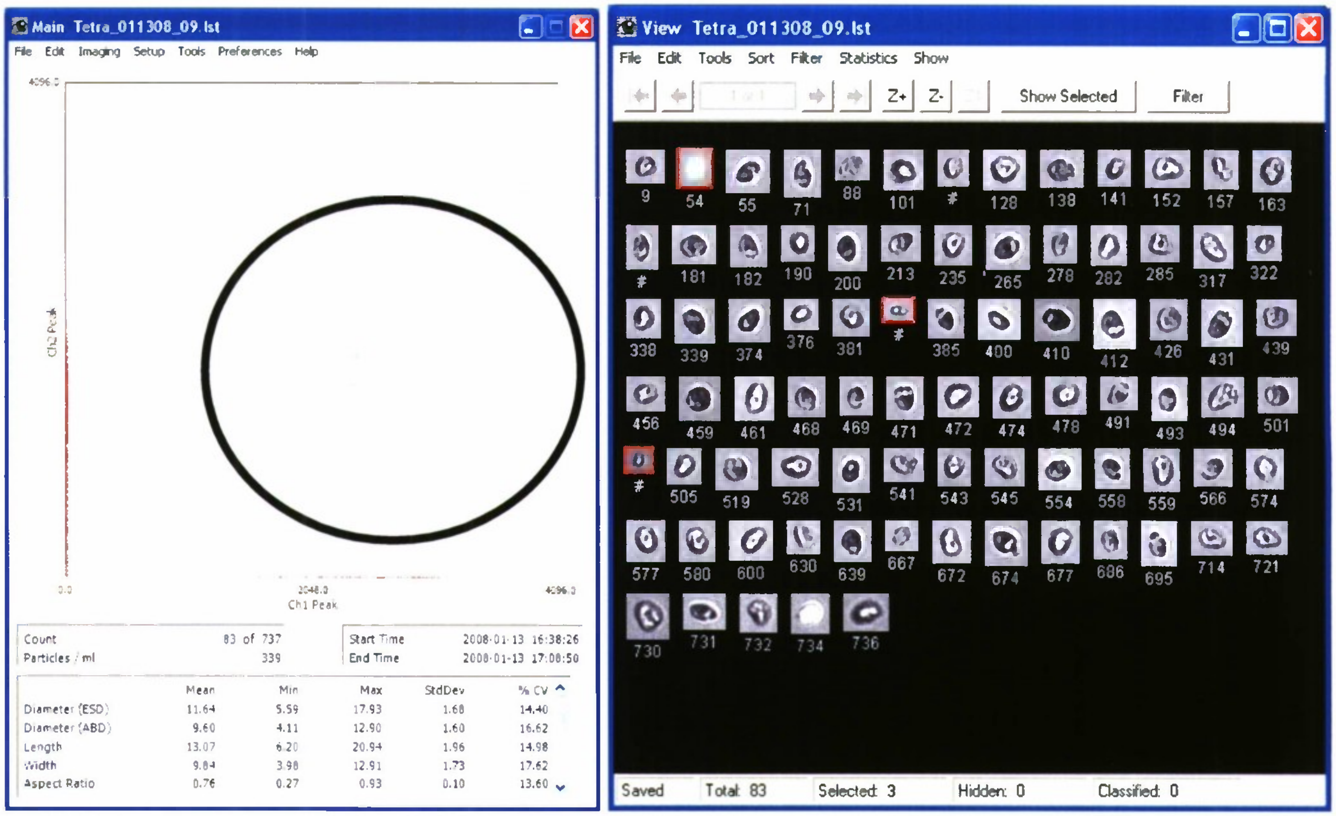

Figure 9. Particles selected had chlorophyll *a* and SYTOX® fluorescence. These particles were classified as non-viable cells

Finally the operator used the Virtual Spreadsheet to select particles with measurable SYTOX® Green fluorescence but no chlorophyll *a* values (Figure 10). In this case 262 particles were reviewed and are displayed in Figure 11. The operator selected particles that appeared to be non-viable phytoplankton cells (i.e., those outlined in red). This step was necessary due to FlowCAM®'s difficiency in reliably measuring chlorophyll *a* fluorescence values, forward scatter, and potentially SYTOX® Green fluorescence. (See Appendix B for more information.)

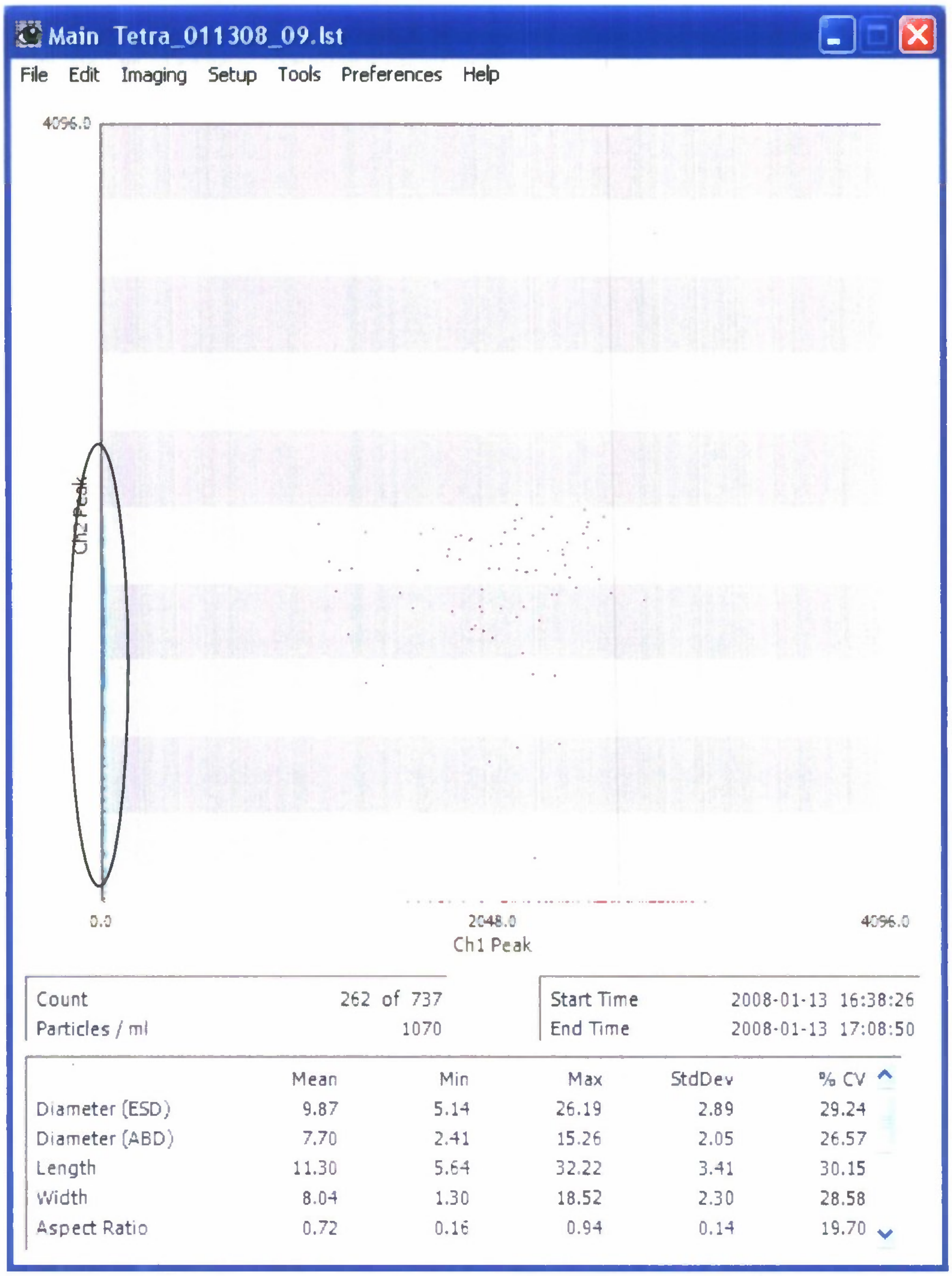

Figure 10. Selected particles have SYTOX® Green but no chlorophyll *a* fluorescence. Some of these particles were classified as non-viable phytoplankton.

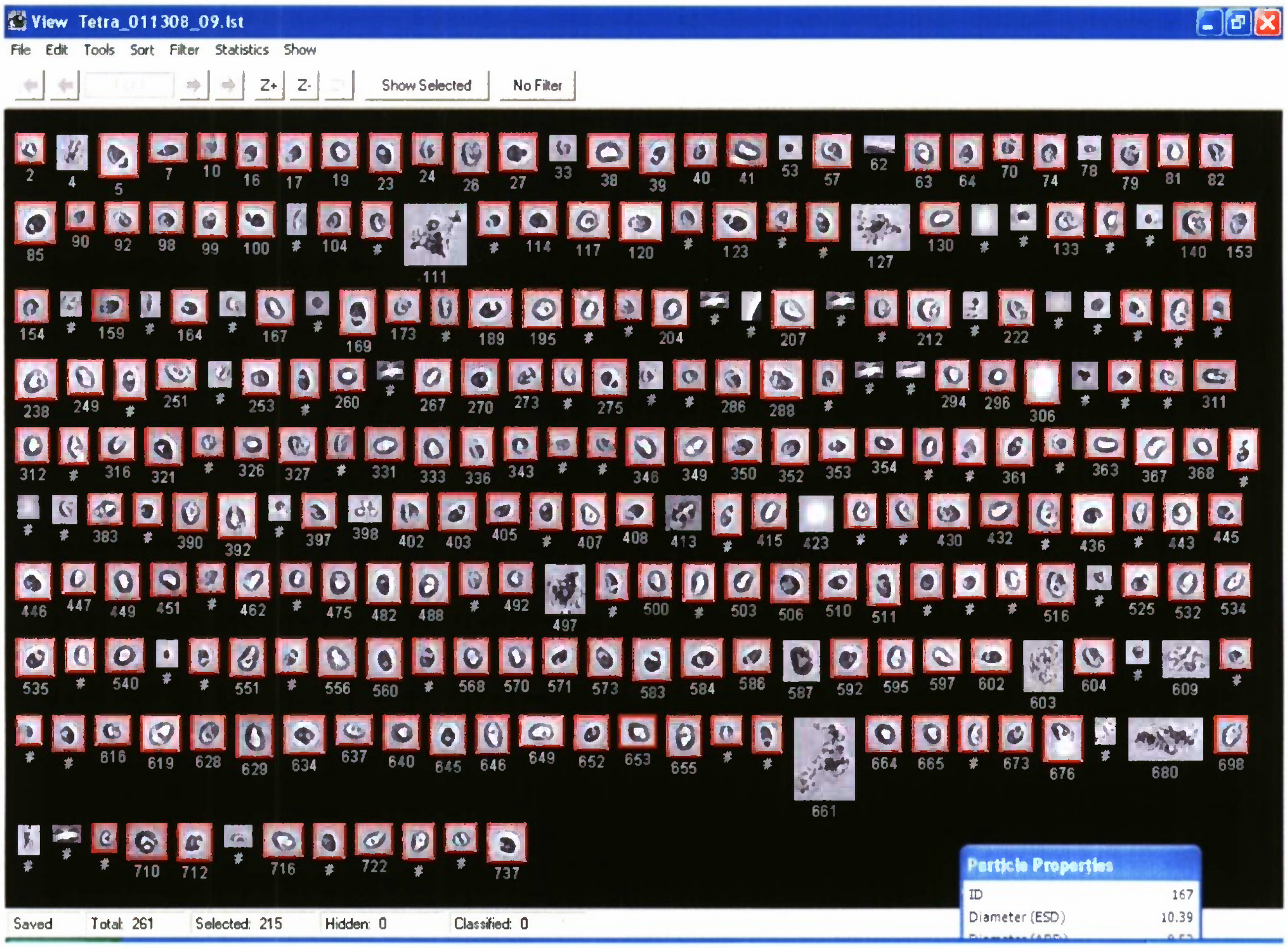

Figure 11. Images associated with particles with SYTOX® fluorescence only. Following a review of the images, 215 of these 261 particles were classified as dead phytoplankton.

The remaining step was to determine whether particles having neither chlorophyll *a* nor SYTOX® Green fluorescence were, in fact, phytoplankton. This involved a manual review of the 298 particles within the ellipse in Figure 12. Most of these particles are shown in Figure 13. In the top panel of the figure, the operator selected 21 phytoplankton-like particles (bordered by red). Visual Spreadsheet pattern recognition software was used to identify particles similar to those selected by the operator. This is possible because the particles do not have potentially erroneous fluorescent values. The images obtained in this manner were then reviewed to remove any that appeared not to be phytoplankton. In this instance 13 additional phytoplankton were identified and are shown in Figure 14 along with the 21 identified by the operator.

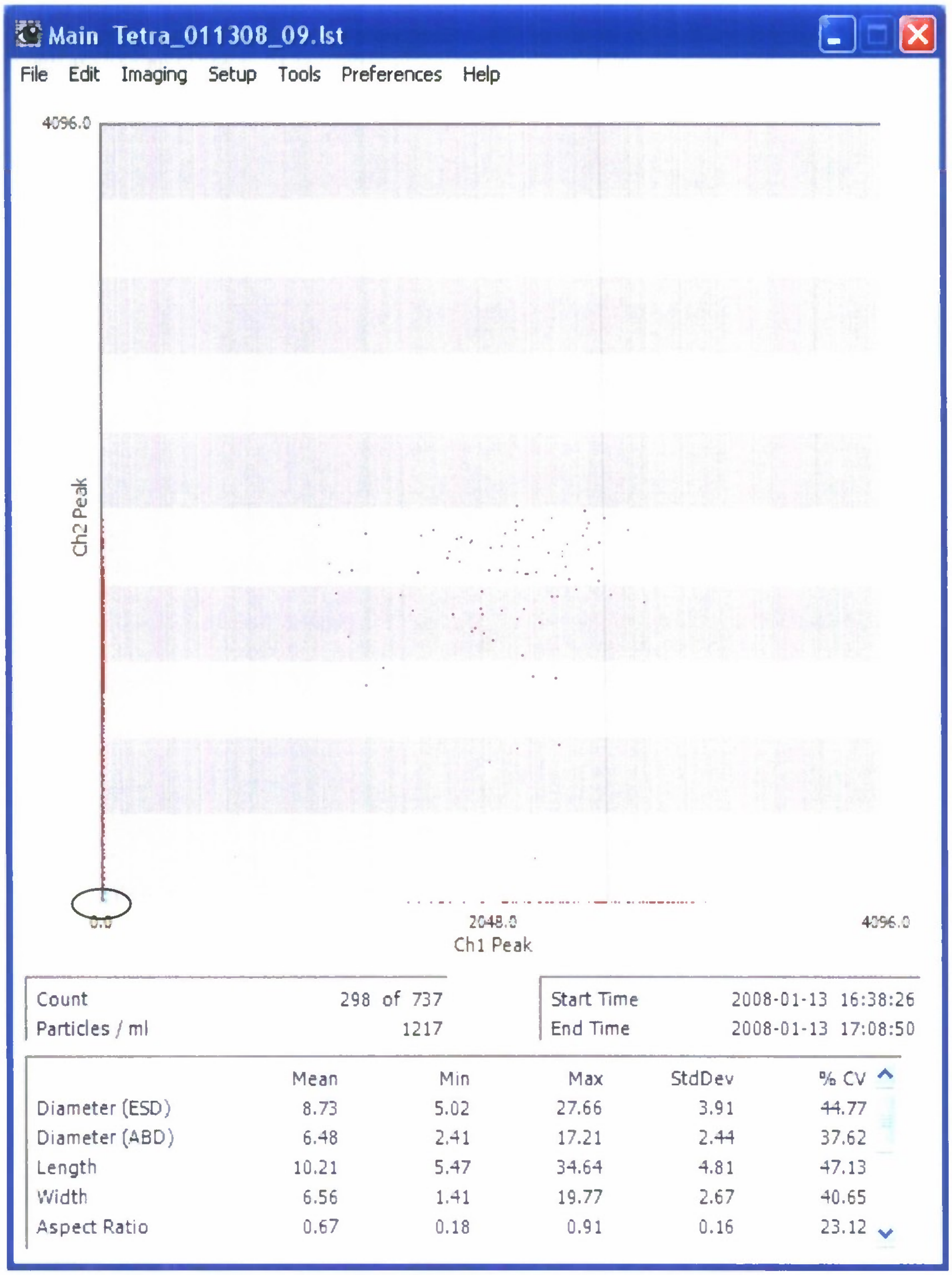

Figure 12. Particles with no measurable chlorophyll *a* or SYTOX® Green fluorescence. Some of these particles were classified as cells of unknown viability.

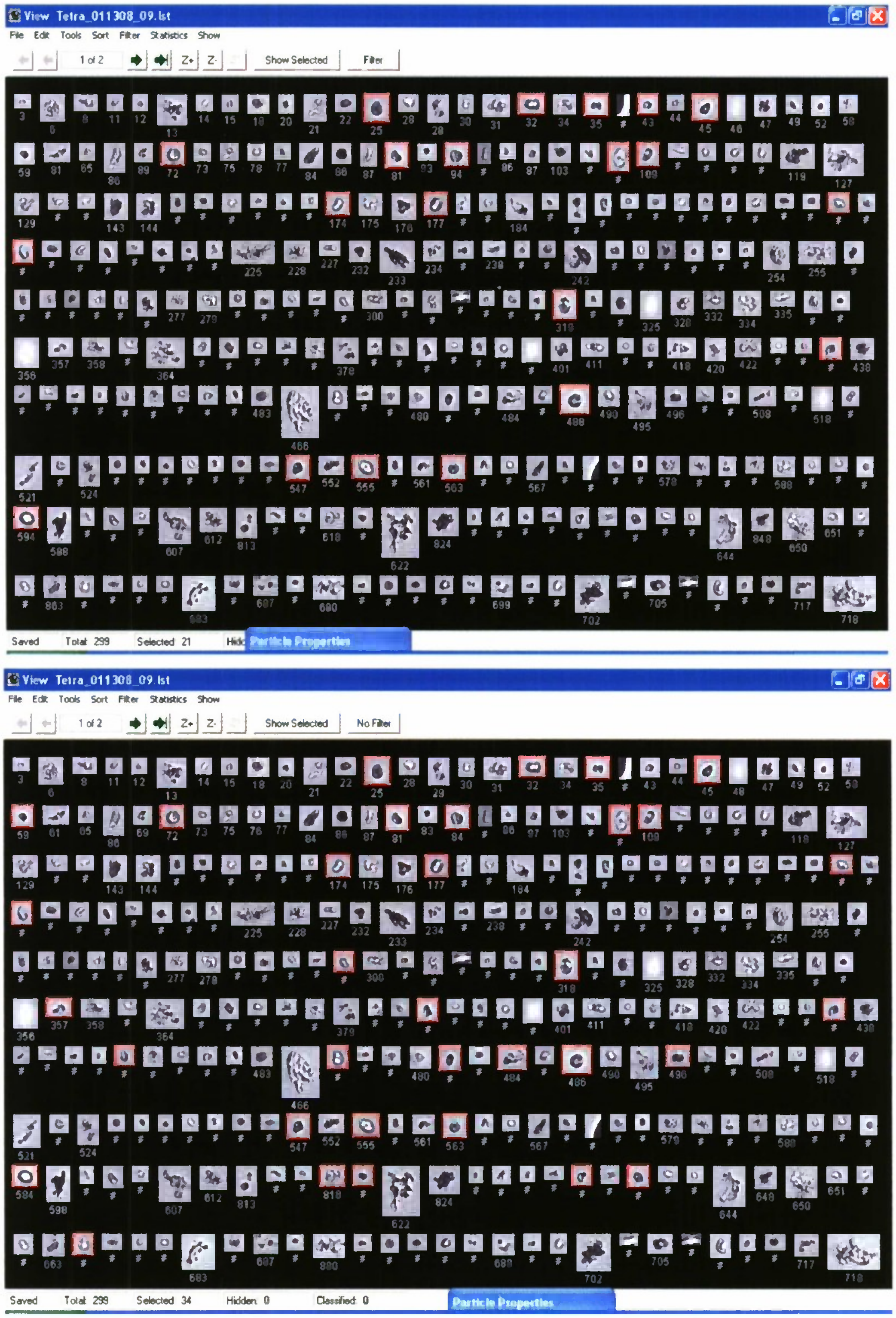

Figure 13. Images of particles with no measurable SYTOX® or chlorophyll *a* fluorescence. Twenty-one particles were selected as potentially being phytoplankton in the upper image. The FlowCAM ® software then selected 13 additional cells with similar properties.

Figure 14. Images of 34 particles that did not have chlorophyll *a* or SYTOX® fluorescence. These particles were classified as cells of unknown viability.

The above steps identified the following for this sample: 92 viable cells, 295 non-viable cells, and 34 +/-10 of unknown viability.

These analysis steps were used for all but the final workshop sample to which test dust had been added. All groups found that SYTOX® Green attached to the test dust such that the stained dust showed the same level of SYTOX® Green fluorescence as phytoplankton cells with compromised membranes (the non-viable phytoplankton). Due to the inconsistent measurement of chlorophyll a fluorescence signal for the previous workshop samples, as FlowCAM is configured, visual inspection of the collected images was the only method to separate SYTOX®-stained test dust from SYTOX®-stained phytoplankton cells.

A second problem from the test dust was that more than one particle was in FlowCAM®'s field of view at any given time. FlowCAM® ascribed the same values to all particles in the field of view which made determination difficult. Because FlowCAM® gave the same value to the test dust and the phytoplankton cells that were viewed together, the final sample was diluted 5:1 and only FlowCAM®'s chlorophyll *a* channel activated. Sample size was 5 mL. Following the run, the images were analyzed to determine the number of phytoplankton cells counted. It was not possible to distinguish between living and dead cells, hence only the total number of phytoplankton cells detected was reported.

For detailed procedures and specific issues and cautions for the FlowCAM® methods, see Appendix B.

2.5.3 Method Advantages and Disadvantages

The major advantage of the FlowCAM® is that it collects images of each particle it detects, thus allowing the operator to verify that detected particles are the result of phytoplankton cells of a particular type. Further, additional information from the detected particle can be extracted from the images using image processing methodologies. This could result in improved means for automating the classification of phytoplankton. It is important to note this capability is not provided in standard flow cytometers. Flow cytometer systems provide fluorescent signal intensity levels for each detected particle (both chlorophyll *a* and SYTOX®) and extract estimates of particle size (equivalent spherical diameters) from the forward scatter signal. They perform these measurements more accurately than the current configuration of the FlowCAM® because the flow cell geometry in flow cytometers results in these measurements being made on single particles always situated in the same position in the flow cell. There is no means, however, to verify that detected particles are truly from phytoplankton cells and that all detected phytoplankton cells are the same species with traditional flow cytometry.

There are several disadvantages of using the FlowCAM® in its current revision to analyze phytoplankton samples. First, the system still does not provide reliable and repeatable measurements of chlorophyll *a* and SYTOX® fluorescence intensity levels or forward scatter intensity levels. This inaccuracy makes it difficult to separate phytoplankton cells from other particles in the sample and also makes it much more difficult to automate the detection and classification (both type and viability) of phytoplankton. Fluid Imaging has recently replaced the forward scatter detector with a detector array which they claim has improved the reliability of forward scatter detection. Additionally, they continue to work on improved measurement algorithms to enhance fluorescence detection. Unfortunately, these improvements were not implemented or available to the FlowCAM® that was used in support of this workshop.

Second, working with non-neutrally buoyant particles can be problematic with the FlowCAM® as a result of its flow cell configuration and its method for introducing samples to this flow cell. A flow cell that allows single particles to be interrogated would be advantageous, but it would likely significantly increase the time required to process a sample, since flow cells of this geometry would have significantly reduced flow rates and consequently slower sample throughput.

Third, a flow cell that allows the entire flow cell's width to be imaged was not available for use with the 20X objective FlowCAM® configuration. Using the FlowCAM® configured with a 10X objective compromised the spatial resolution of the particles' images and rendered it difficult—using either manual or automated methods—to unambiguously differentiate phytoplankton from test dust and other particles.

In response to the deficiencies mentioned above and in support of the Phytoplankton Enumeration and Evaluation Workshop, NRLKW and Fluid Imaging Technologies developed methods that helped obviate some of the deficiencies in the DSP-based FlowCAM®. Because the "automated" and rapid analysis capabilities of the FlowCAM® could not be utilized, significant manual analysis time was required to process the data. It is believed that the methodologies developed overcame the DSP-based FlowCAM®'s deficiency in providing reliable and consistent fluorescence and forward scatter intensity measurements. If these measurements could be made more reliable, then it is likely that analysis of phytoplankton samples using the FlowCAM® could be performed more rapidly with high efficiencies.

The identified deficiency with the FlowCAM®'s current fluidic design and non-neutrally buoyant particles, however, should preclude its use in analyzing BWTE samples that have added mineral matter or other non-neutrally buoyant particles.

3 RESULTS AND DISCUSSION

The results of analysis of individual samples for each of the analytical methods conducted at the workshop are presented below. This allows sample-to-sample and method-to-method comparisons. The final sub-section provides a comparison of methods.

3.1 Sample

Samples were prepared according to the procedures outlined in Section 2.1. Table 3 provides a summary of the target Live/Dead sample concentrations for the seven workshop test samples. As mentioned previously, Sample 7 had TOC, POM, and MM added to it and the concentrations were 10 times higher than that typically encountered in BWTE tests at NRLKW.

Table 3. Workshop target concentrations and viability ratios.

Sample #	Sample Date	Sample Time	Reed Mariculture Stock Count (total cells mL^{-1})	Date Stock Received at NRLKW	Target Concentrations (Live/Dead mL^{-1})
1	11-Jan	10:00	708,750	10-Jan	30/300
2	11-Jan	14:00	708,750	10-Jan	100/1000
3	12-Jan	10:00	708,750	10-Jan	141/141
4	12-Jan	14:00	858,750	12-Jan	20/20
5	13-Jan	10:00	858,750	12-Jan	20/200
6	13-Jan	14:00	858,750	12-Jan	20/1000
7	14-Jan	14:00	858,750	12-Jan	20 (live only)

Although the workshop took place January 6 – 16, samples for the first 3 days of the workshop were considered preliminary tests used to fine tune methodologies employed for live- and dead-cell enumeration. Beginning on January 11, 2008, the data collected from each of the methods were compared to one another, and a brief synopsis of each of the samples analyzed using the various methods is presented below.

3.2 NRLKW - Microscopy

3.2.1 10 AM Sample from January 11, 2008 – Target Test Sample Concentration 300 Dead: 30 Live Cells

Figure 15 provides results from the 10 AM Sample from January 11, 2008. The target concentration for this sample (Sample 1) was 30 live cells: 300 dead cells mL^{-1}. The mean for the three subsamples was 173 dead cells mL^{-1} and 26 live cells mL^{-1}. Both the live and dead cell concentrations were within a factor of two of the target concentrations. ("Factor of two" implies the sample value is between 1/2x target and 2x the target values.) The results obtained by the NRLKW microscopy team were consistent with those of the other workshop methods.

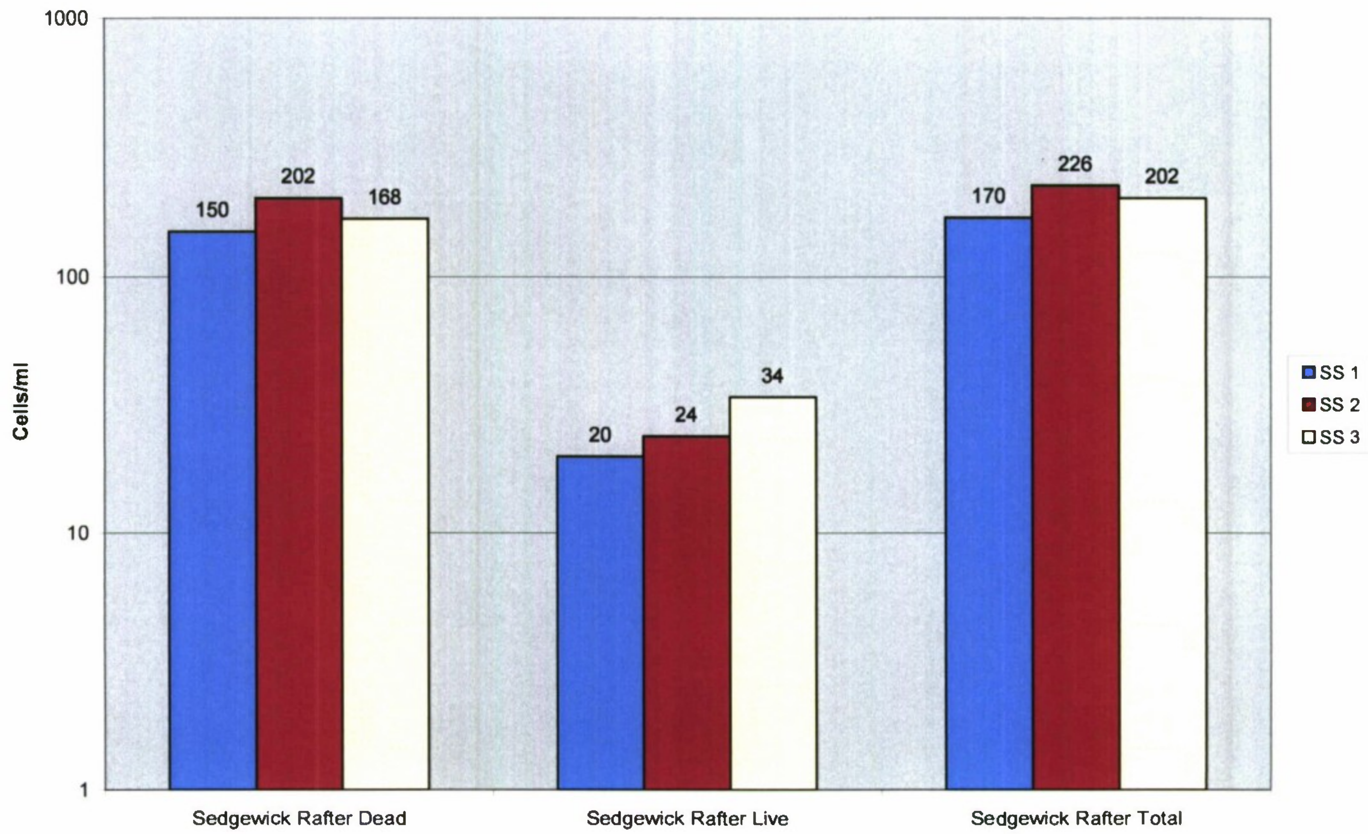

Figure 15. Sedgwick Rafter counts for the 10 AM January 11, 2008 workshop sample.

3.2.2 2:00 PM Sample from January 11, 2008 – Target Test Sample Concentration 1000 Dead: 100 Live Cells

Figure 16 provides results from the 2 PM Sample from January 11, 2008. The target concentration for this sample was 100 live cells: 1000 dead cells mL^{-1}. The mean for the three subsamples was 259 dead cells mL^{-1} and 53 live cells mL^{-1}. The live cell concentrations were within a factor of two of the target concentrations for this sample. The dead cell concentration, however, was considerably lower than the target concentration. There is, however, fairly close consistency among the three 1-mL subsamples that were analyzed.

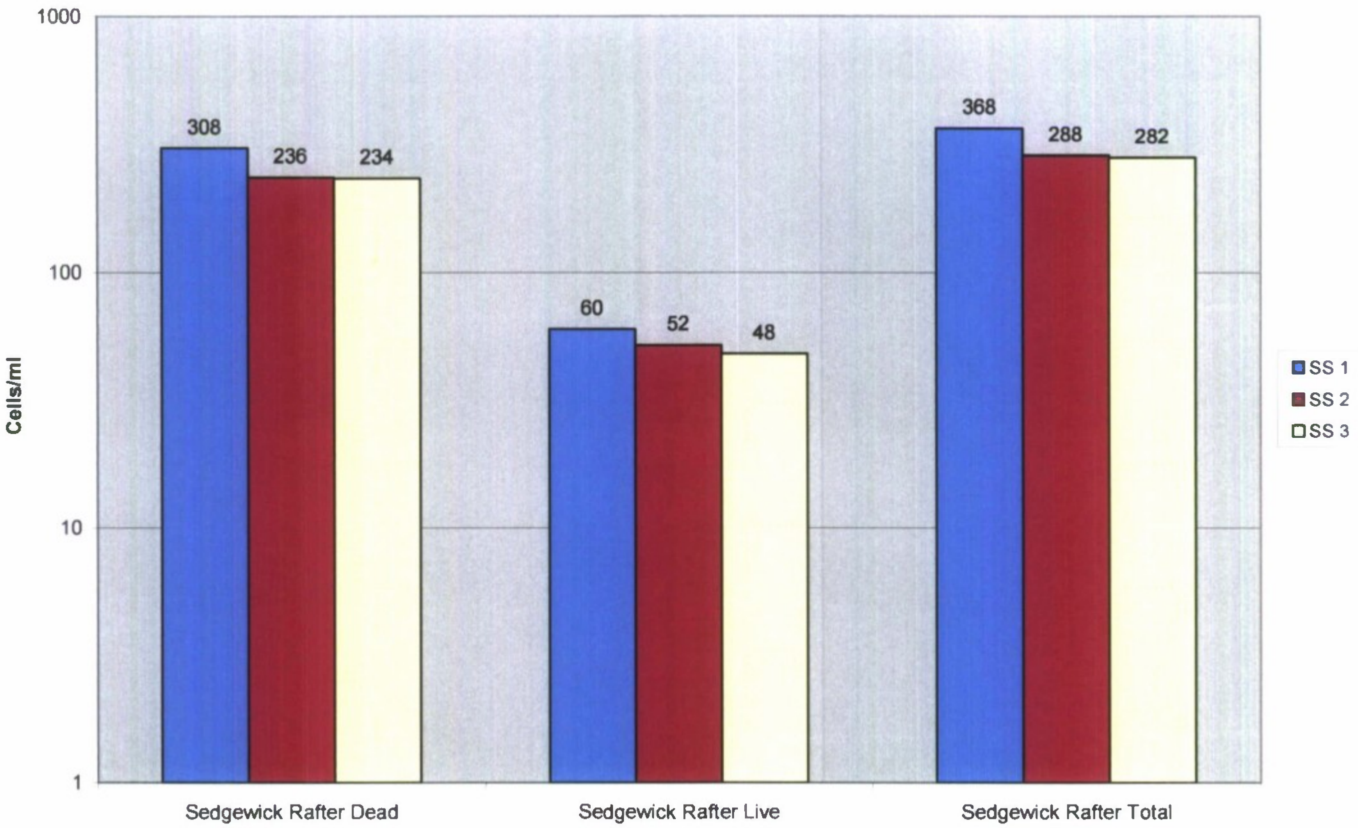

Figure 16. Sedgwick Rafter counts for the 2 PM January 11, 2008 workshop sample.

3.2.3 10:00 AM Sample from January 12, 2008 – Target Test Sample Concentration 141 Dead: 141 Live Cells

Figure 17 provides results from the 10 AM Sample from January 12, 2008. The target concentration for this sample was 141 live cells: 141 dead cells mL^{-1}. The mean for the three sample subsamples was 145 dead cells mL^{-1} and 32 live cells mL^{-1}. For this sample the dead cell concentrations were well within a factor of two of the target concentrations. The live cell concentrations, however, were almost a factor of four lower than the target concentration. With the exception of the live concentration for subsample 2, the consistency between the three subsamples analyzed was similar to that seen for the previous sample.

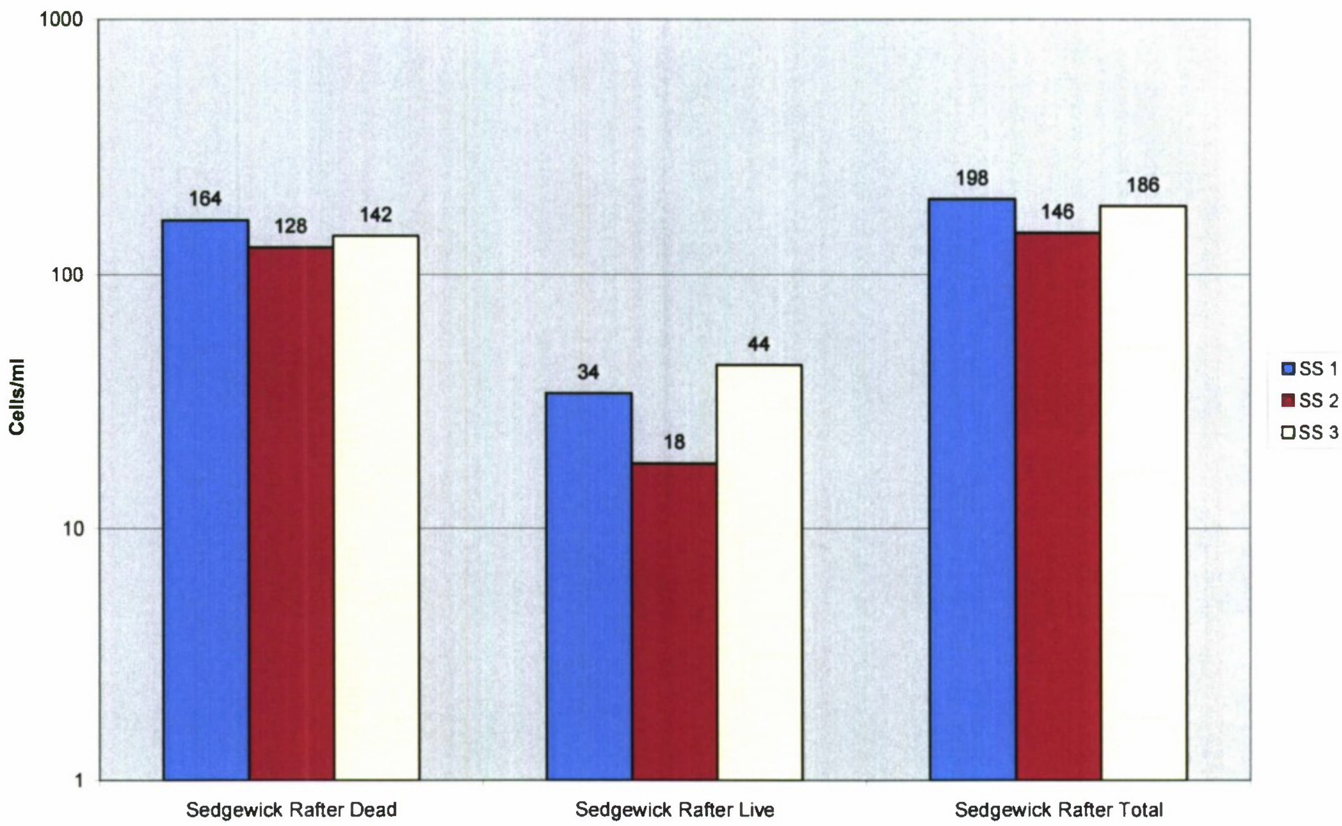

Figure 17. Sedgwick Rafter counts for the 10 AM January 12, 2008 workshop sample.

3.2.4 2:00 PM Sample from January 12, 2008 – Target Test Sample Concentration 20 Dead: 20 Live Cells

Figure 18 provides results from the 2 PM Sample from January 12, 2008. Note the scale change from the previous figures. The target concentration for this sample was 20 live cells: 20 dead cells mL^{-1}. The mean for the three subsamples was 10 dead cells mL^{-1} and 19 live cells mL^{-1}. Although the value for dead cells for subsample 3 is less than half the value of the other subsamples, both the live and dead cell concentrations for all other subsamples were within a factor of two of the target concentrations for this sample.

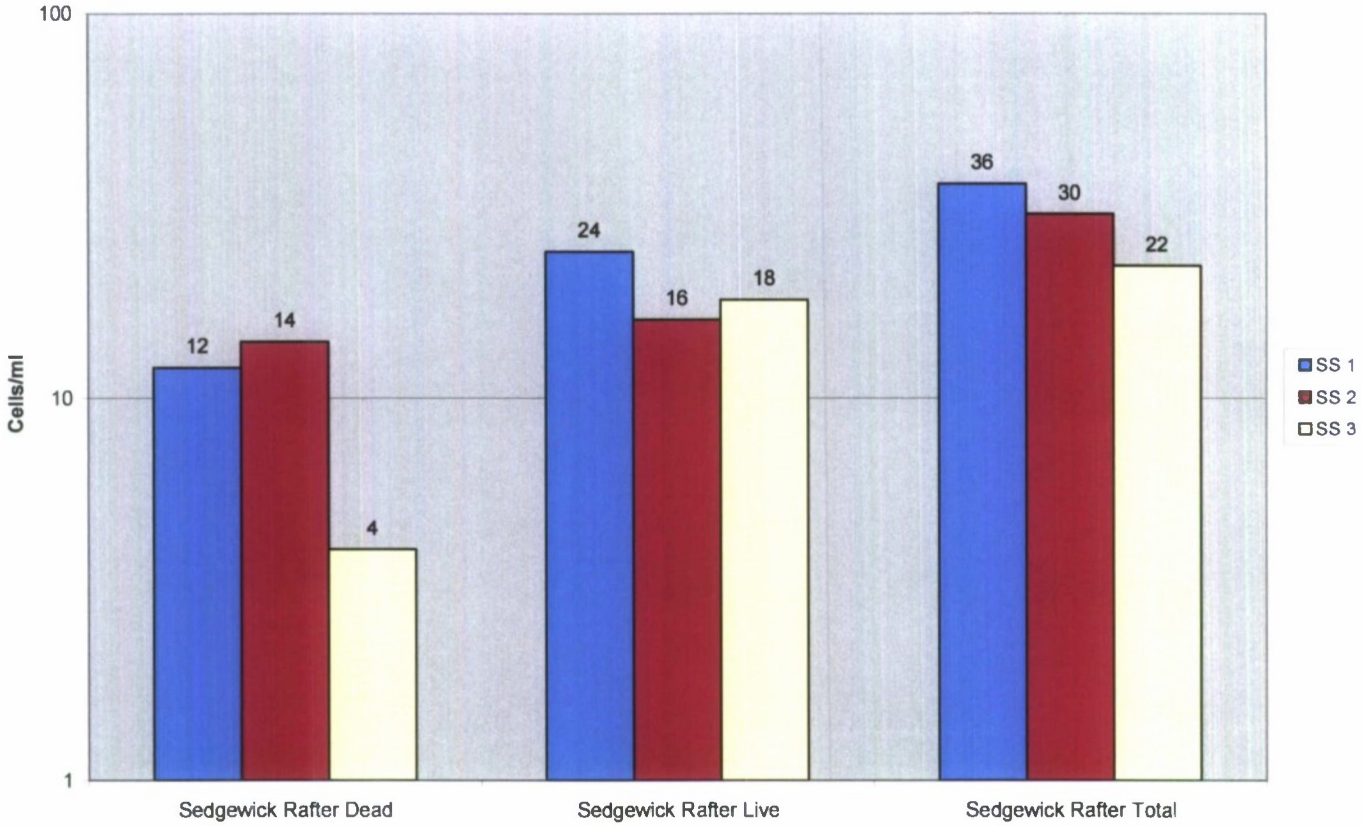

Figure 18. Sedgwick Rafter counts for the 2 PM January 12, 2008 workshop sample.

3.2.5 10:00 AM Sample from January 13, 2008 – Target Test Sample Concentration 200 Dead: 20 Live Cells

Figure 19 provides results from the 10 AM Sample from January 13, 2008. The target concentration for this sample was 20 live cells: 200 dead cells mL^{-1}. The mean for the three subsamples was 77 dead cells mL^{-1} and 23 live cells mL^{-1}. The live and dead cell concentrations were within a factor of three of the target concentrations for this sample.

While there was strong consistency among subsamples, the results reported for the dead cell concentrations in this sample were higher than those reported by the other workshop participants for this sample.

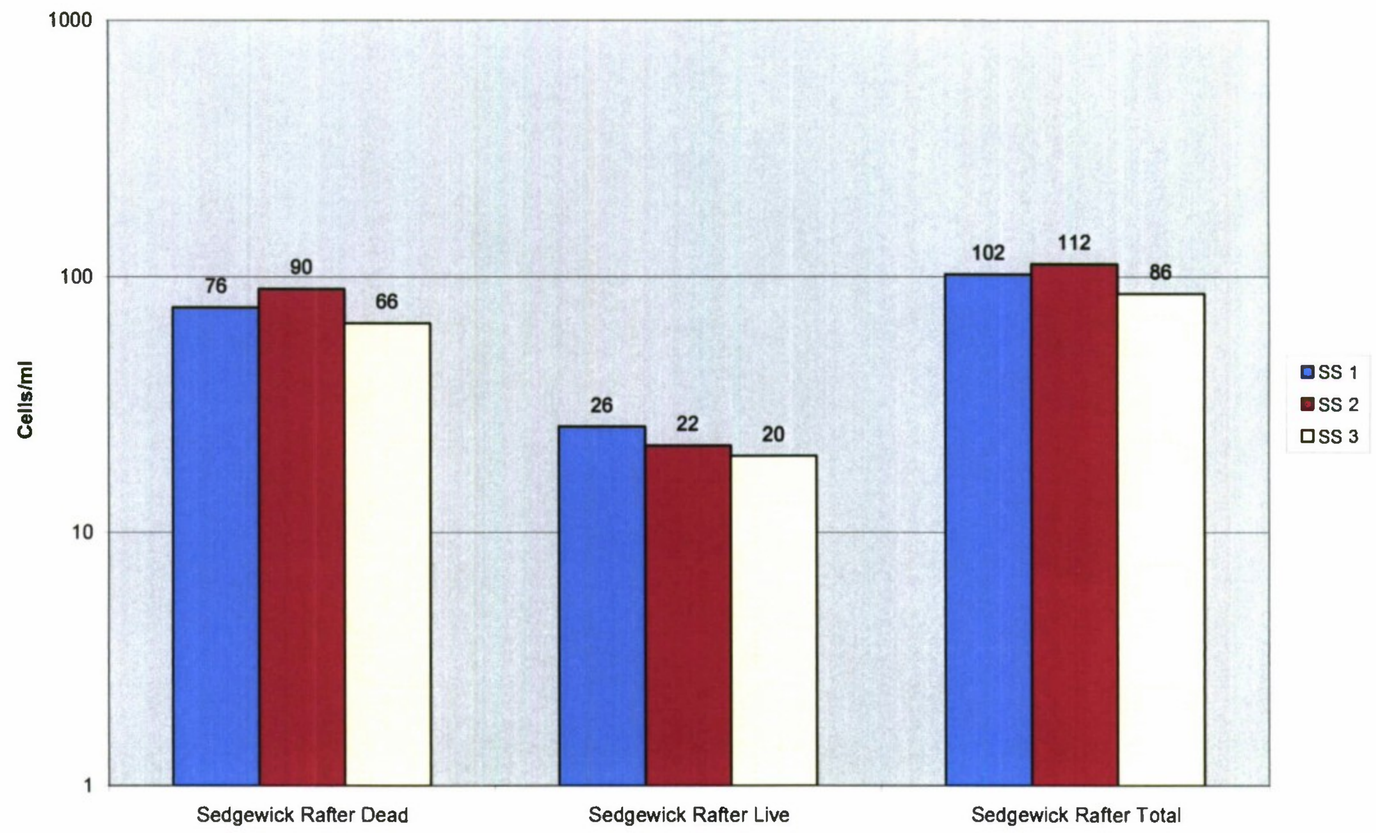

Figure 19. Sedgwick Rafter counts for the 10 AM January 13, 2008 workshop sample.

3.2.6 2:00 PM Sample from January 13, 2008 – Target Test Sample Concentration 1000 Dead: 20 Live Cells

Figure 20 provides results from the 2 PM Sample from January 13, 2008. The target concentration for this sample was 20 live cells: 1000 dead cells mL^{-1}. The mean for the three subsamples was 449 dead cells mL^{-1} and 17 live cells mL^{-1}. The dead cell concentrations are nearly within a factor of two of target concentrations for this sample.

The live cell concentrations are much closer. The live cell concentration reported for this sample was lower than those reported by the other workshop participants. Both microscopy groups (NRLKW and WHOI) reported difficulty in working with this sample.

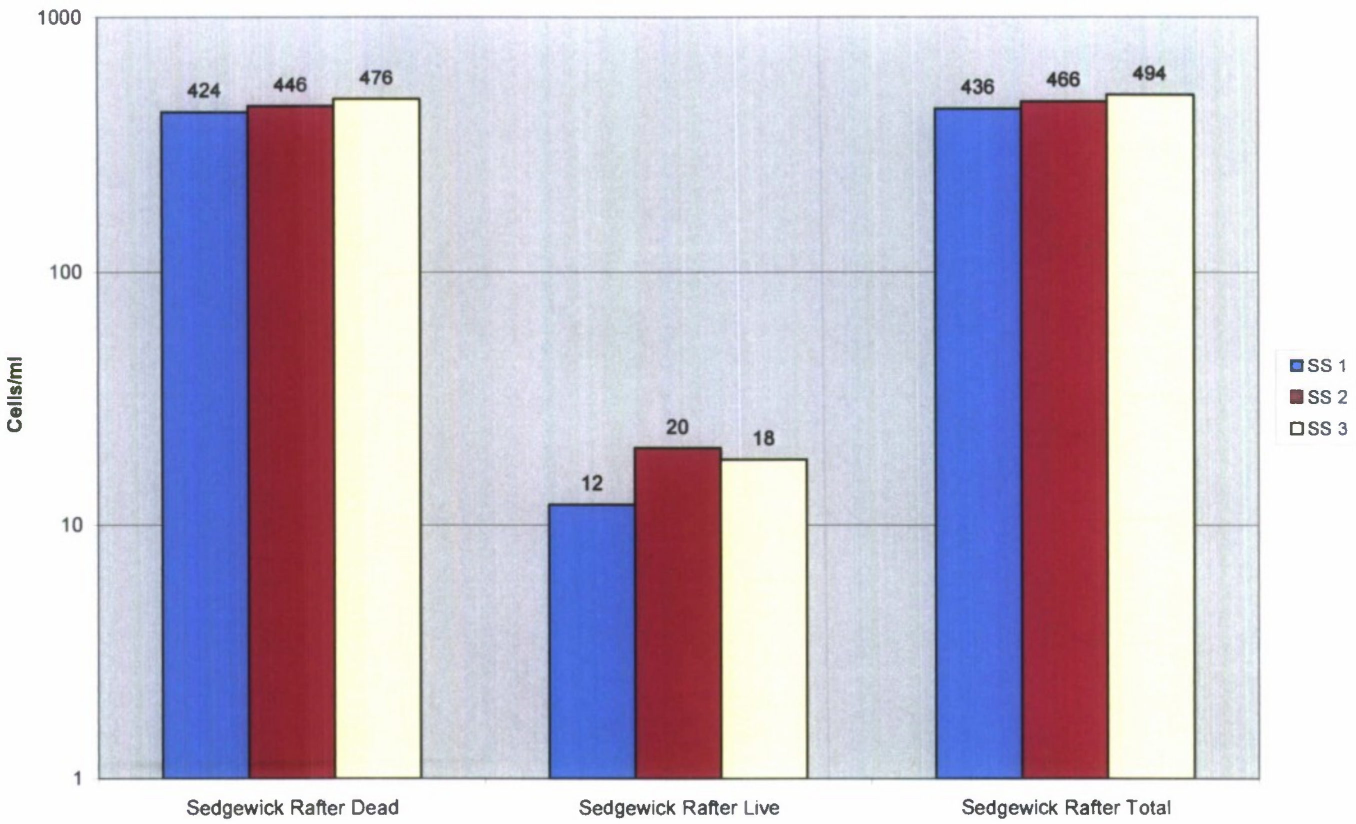

Figure 20. Sedgwick Rafter counts for the 2 PM January 13, 2008 workshop sample.

3.2.7 Sample from January 14, 2008 – Target Test Sample Concentration 20 Live Cells With TSS and TOC Added

Figure 21 provides results from the complex sample from January 14, 2008. The target concentration for this sample was 20 live cells, and the sample had TOC, POM and MM added to make the sample representative of a BWTTF water sample. Note that the target number of dead cells for this sample was not known as only live cells were added. As can be seen in the Figure, the mean for the three subsamples was 19 dead cells mL^{-1} and 53 live cells mL^{-1}. The live cell concentration is within a factor of 3 of the expected value for this sample. There is also reasonably good consistency for live and total cell counts across the three 1 mL subsamples that were analyzed from this workshop sample. This is especially true for the live cell concentrations.

The complexity of this sample had little impact on the ability of NRLKW researchers to efficiently characterize this sample.

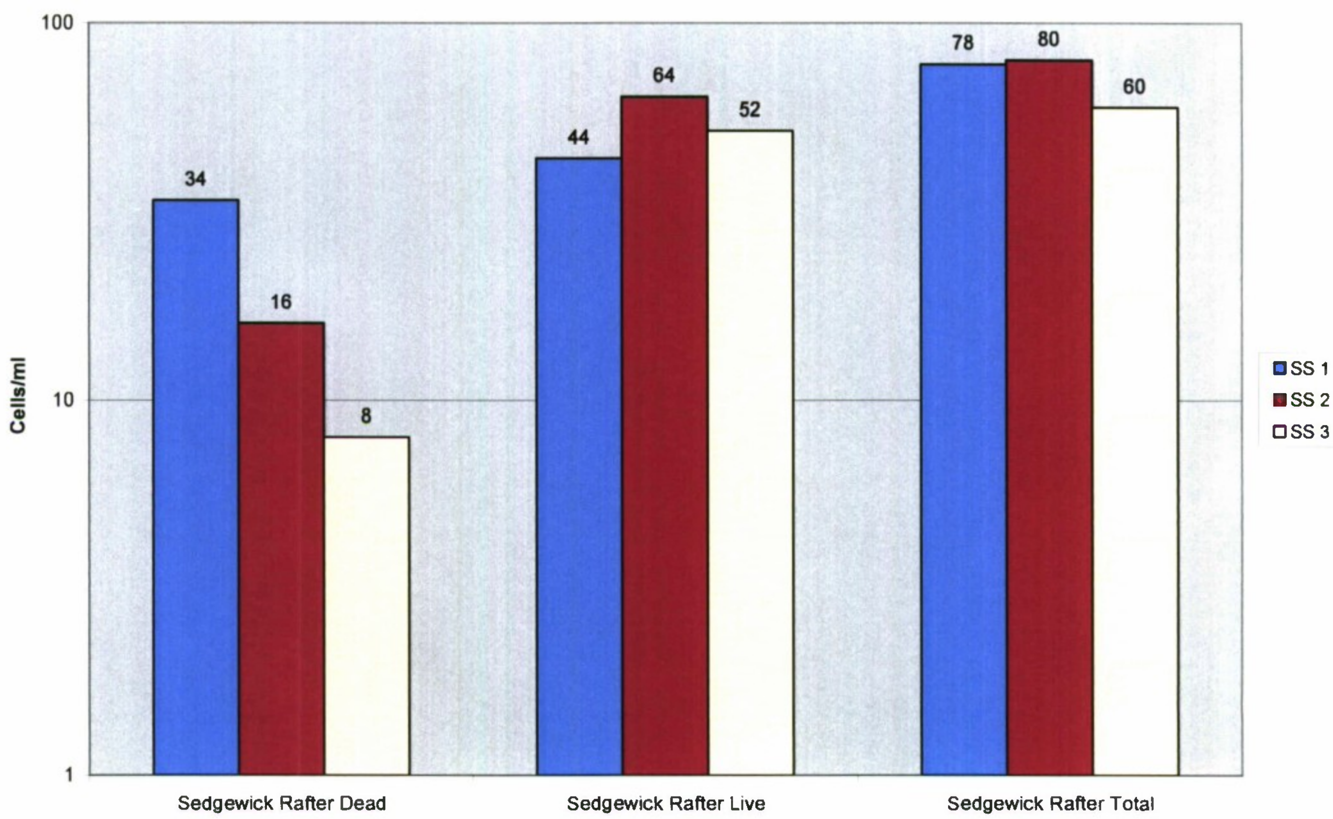

Figure 21. Sedgwick Rafter counts for the January 14, 2008 workshop sample.

3.3 WHOI – Microscopy

3.3.1 10 AM Sample from January 11, 2008 – Target Test Sample Concentration 300 Dead: 30 Live Cells

Two samples each were enumerated for both SYTOX® Green and Cell Tracker™ Green CMFDA vital stained samples. In all cases, only half of the filter was counted for each subsample, and no problems were encountered during microscopic enumeration. The results are provided in Figure 22.

The SYTOX® Green and Cell Tracker™ Green CMFDA values for live and dead cell totals in this sample complemented each other and were similar to the expected concentrations of 300 dead and 30 live cells (Figure 22). For this sample, the means were 155 dead cells mL^{-1} and 30 live cells mL^{-1} using SYTOX® and 205 dead cells mL^{-1} and 22 live cells mL^{-1} using Cell Tracker™ Green CMFDA.

The reported results were within a factor of two of the target numbers and were consistent with those reported by the other workshop participants.

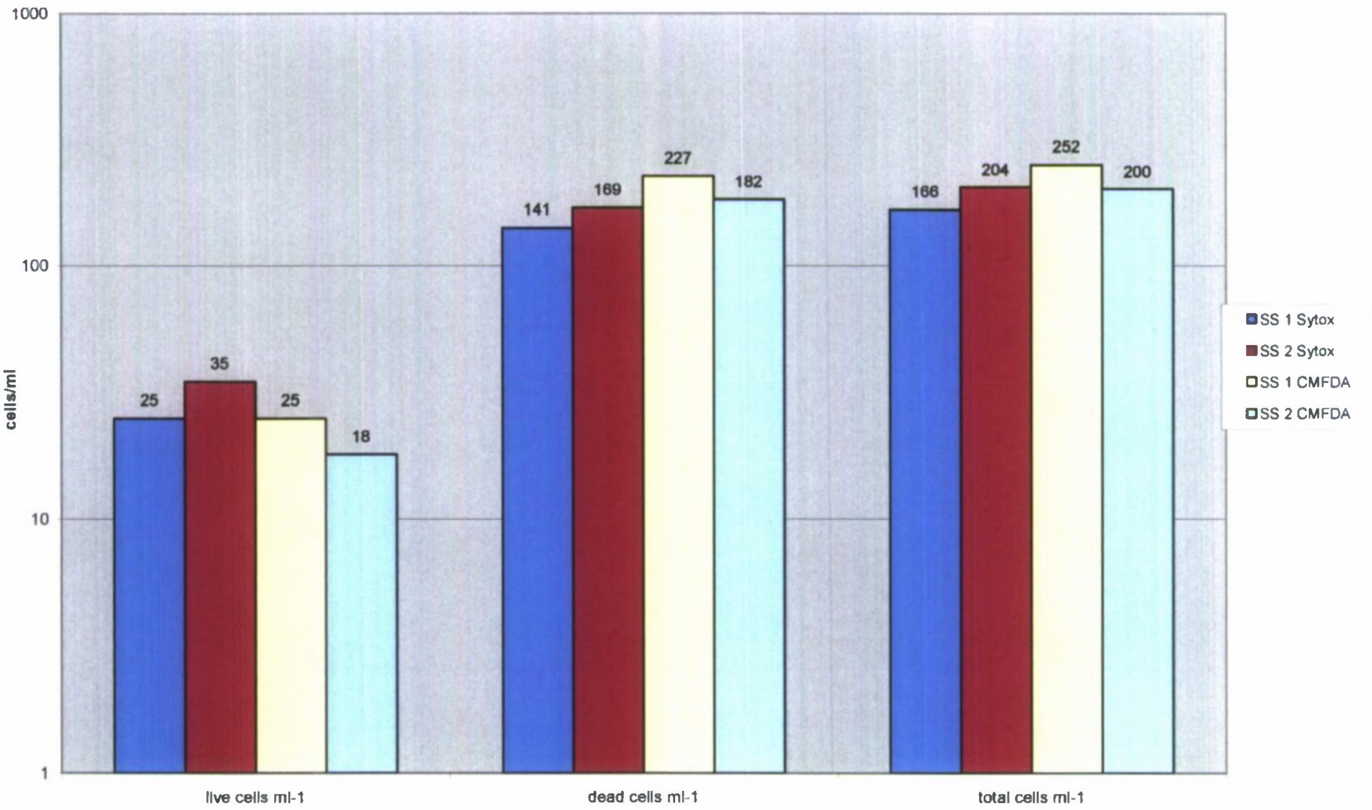

Figure 22. Filtration/microscopy counts For SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 10 AM January 11, 2008 workshop sample.

3.3.2 2:00 PM Sample from January 11, 2008 – Target Test Sample Concentration 1000 Dead: 100 Live Cells

A one-liter sample was supplied and three 5-mL subsamples were processed for this sample. Two samples each were enumerated for both SYTOX® Green and Cell Tracker™ Green CMFDA stained samples, and again, in all cases, only half of the filter was counted for each subsample. No problems were encountered during microscopic enumeration. The results are shown in Figure 23.

The SYTOX® Green and Cell Tracker™ Green CMFDA values for live and dead cell totals in this sample complemented each other; only the live values were similar to the expected concentrations however. The results showed means of 181 dead cells mL^{-1} and 71 live cells mL^{-1} using SYTOX® and 193 dead cells mL^{-1} and 79 live cells mL^{-1} using Cell Tracker™ Green CMFDA. Consistency was excellent across the four subsamples analyzed.

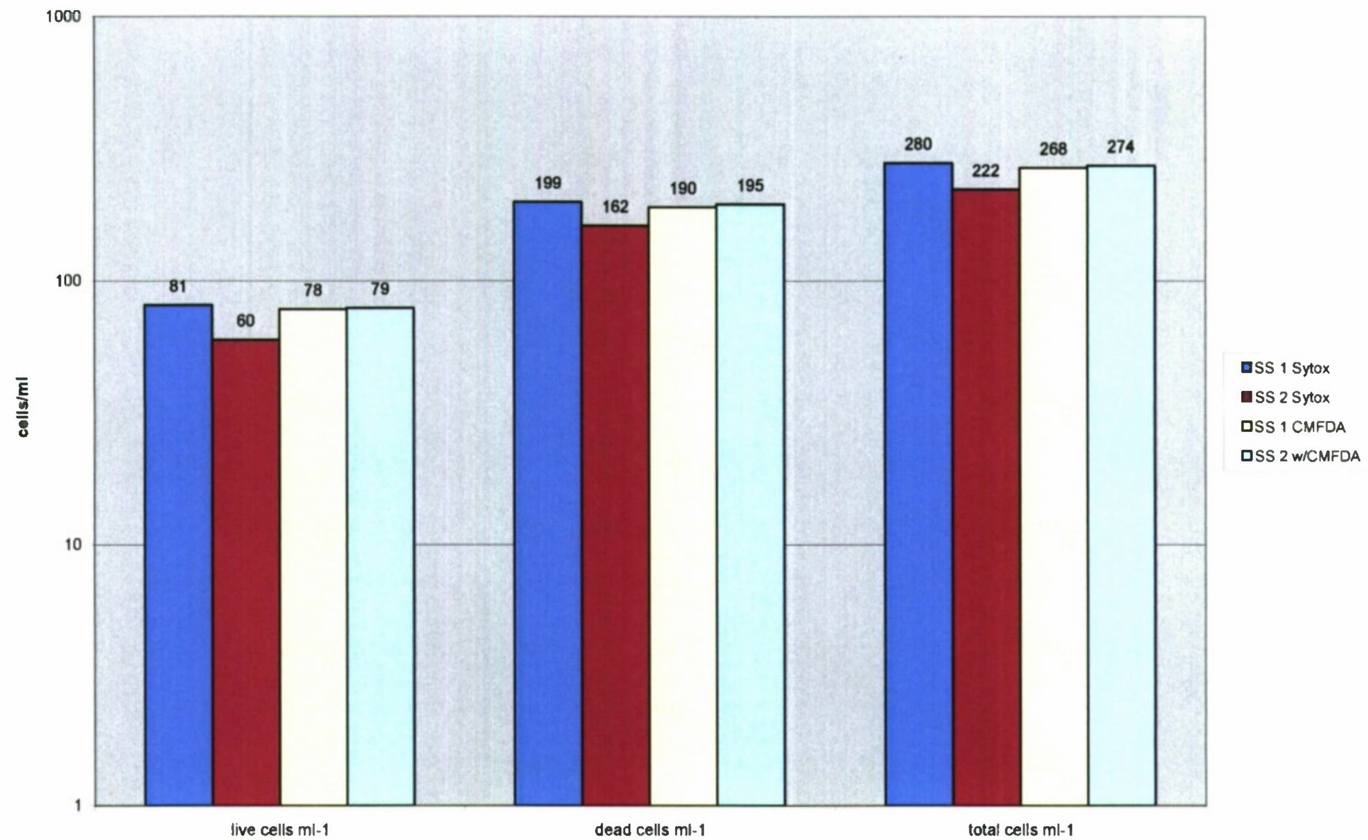

Figure 23. Filtration/microscopy counts for SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 2 PM January 11, 2008 workshop sample.

3.3.3 10:00 AM Sample from January 12, 2008 – Target Test Sample Concentration 141 Dead: 141 Live Cells

A 300 mL sample was supplied and three 5-mL subsamples were processed for this sample. Three samples were each enumerated for both SYTOX® Green and for Cell Tracker™ Green CMFDA vital stains for a total of six samples analyzed. In all cases, only half of the filter was counted for each subsample, and no problems were encountered during microscopic enumeration. The results are shown in Figure 24.

For this sample, the means were 40 dead cells mL^{-1} and 87 live cells mL^{-1} using SYTOX® and 98 dead cells mL^{-1} and 100 live cells mL^{-1} using Cell Tracker™ Green CMFDA. The SYTOX® Green and Cell Tracker™ Green CMFDA values in this sample complemented each other for the live cell concentrations but were contradictory for the dead cell concentrations. The results for live cell concentrations were within a factor of two of the target numbers. The dead cell concentrations reported for the SYTOX® method were within a factor of four of the target concentration while much better agreement was found with the CMFDA method. Given that the *Tetraselmis* cells in all of the samples are very easy to identify and count due to their bright chlorophyll *a* fluorescence, it is believed that the lower cell numbers in the SYTOX® Green sample were due to subsample variability and not the stain's failure to work properly. As discussed earlier, results for this sample concentration were less consistent among the various methods used by the workshop participants than for other samples.

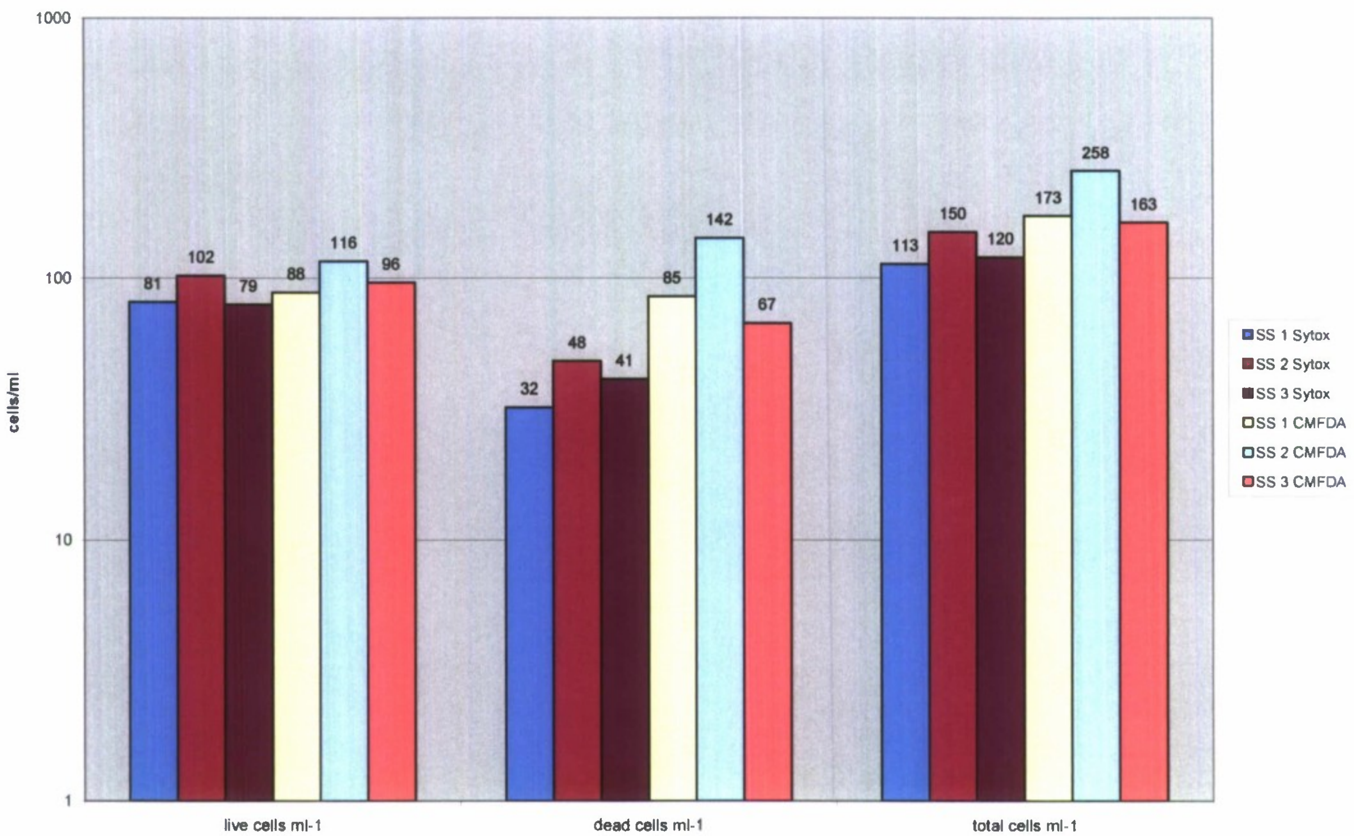

Figure 24. Filtration/microscopy counts for SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 10 AM January 12, 2008 workshop sample.

3.3.4 2:00 PM Sample from January 12, 2008 – Target Test Sample Concentration 20 Dead: 20 Live Cells

A 300 mL sample was supplied and six 10-mL subsamples were processed for this sample. Three samples each were enumerated for SYTOX® Green and three more for Cell Tracker™ Green CMFDA vital stained samples. In all cases, the complete filter was counted for each subsample. No problems were encountered during microscopic enumeration. The results are presented in Figure 25.

The SYTOX® Green and Cell Tracker™ Green CMFDA values for live and dead cell totals in this sample complemented each other. The dead cell concentrations were within a factor of two of the target concentrations while the live cell concentrations were within a factor of three. The means were 15 live cells mL^{-1} and 6 dead cells mL^{-1} using SYTOX® Green and 14 live cells mL^{-1} and 7 dead cells mL^{-1} using Cell Tracker™ Green CMFDA.

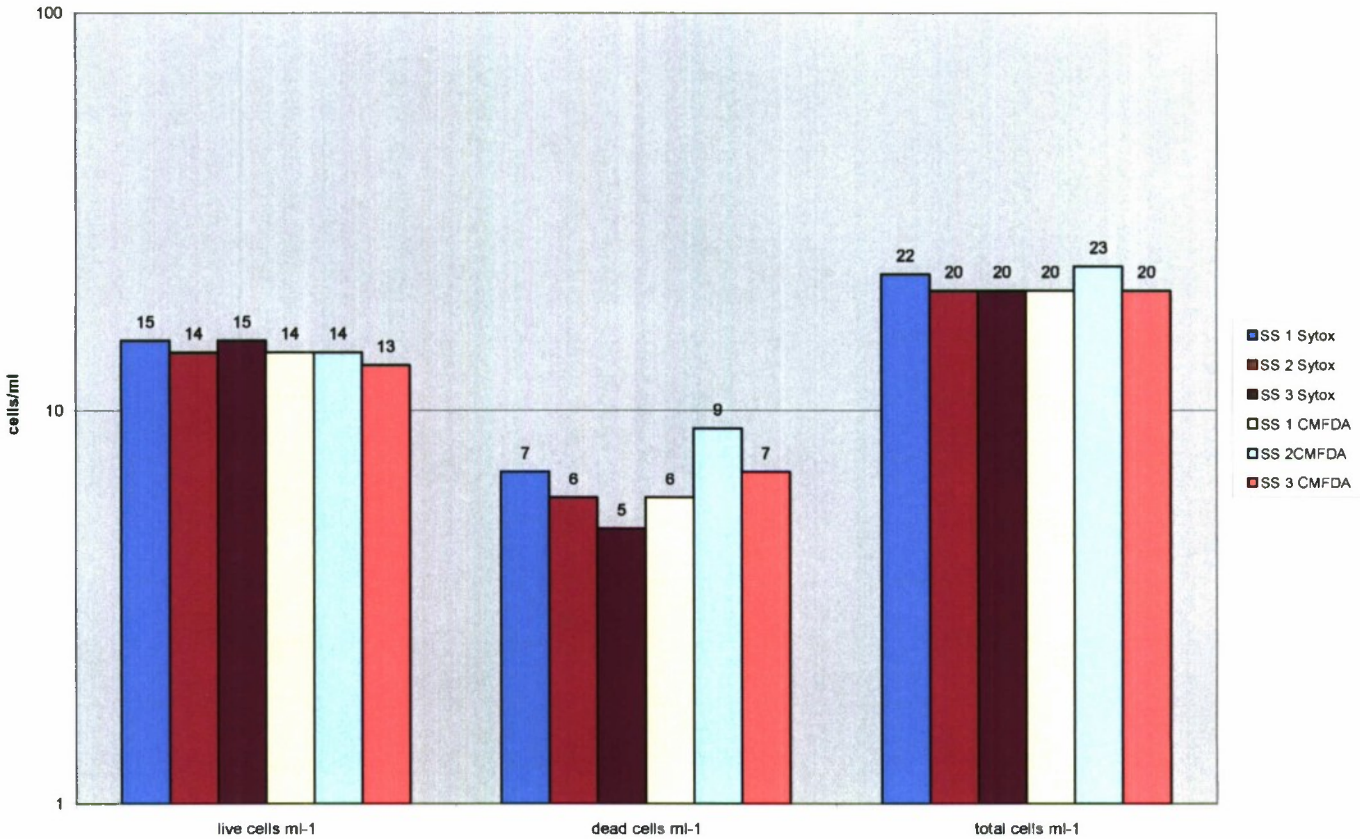

Figure 25. Filtration/microscopy counts For SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 2 PM January 12, 2008 workshop sample.

3.3.5 10:00 AM Sample from January 13, 2008 – Target Test Sample Concentration 200 Dead: 20 Live Cells

A 300 mL sample was supplied and six 5-mL subsamples were processed for this workshop sample. Three samples were each enumerated for SYTOX® Green and three for Cell Tracker™ Green CMFDA vital stains. In all cases, the complete filter was counted for each subsample, and no problems were encountered during microscopic enumeration. The Cell Tracker™ Green CMFDA labeled cells in this sample did not seem to be as bright as those witnessed in the previous sample sets. This effect may be a result of degradation of the DMSO used to re-suspend the stain prior to its use. This hypothesis is supported by the results obtained on January 14, 2008 when fresh DMSO was used to re-suspend the Cell Tracker™ Green CMFDA. In that instance, more consistent, brighter cell labeling was evident.

The mean numbers of stained cells in this sample were 55 live cells mL^{-1} and 22 dead cells mL^{-1} using SYTOX® Green and 27 live cells mL^{-1} and 51 dead cells mL^{-1} using Cell Tracker™ Green CMFDA (Figure 26). The dead cell concentrations for both stains were significantly lower than the target dead cell concentrations for this workshop sample. The live cell concentrations are more consistent with the target live cell concentrations. The live count for SYTOX® Green was within a factor of three of the target concentration while the live count for Cell Tracker™ Green CMFDA was within a factor of two. Both the live and dead cell concentrations were similar to the results for the other workshop enumeration methods.

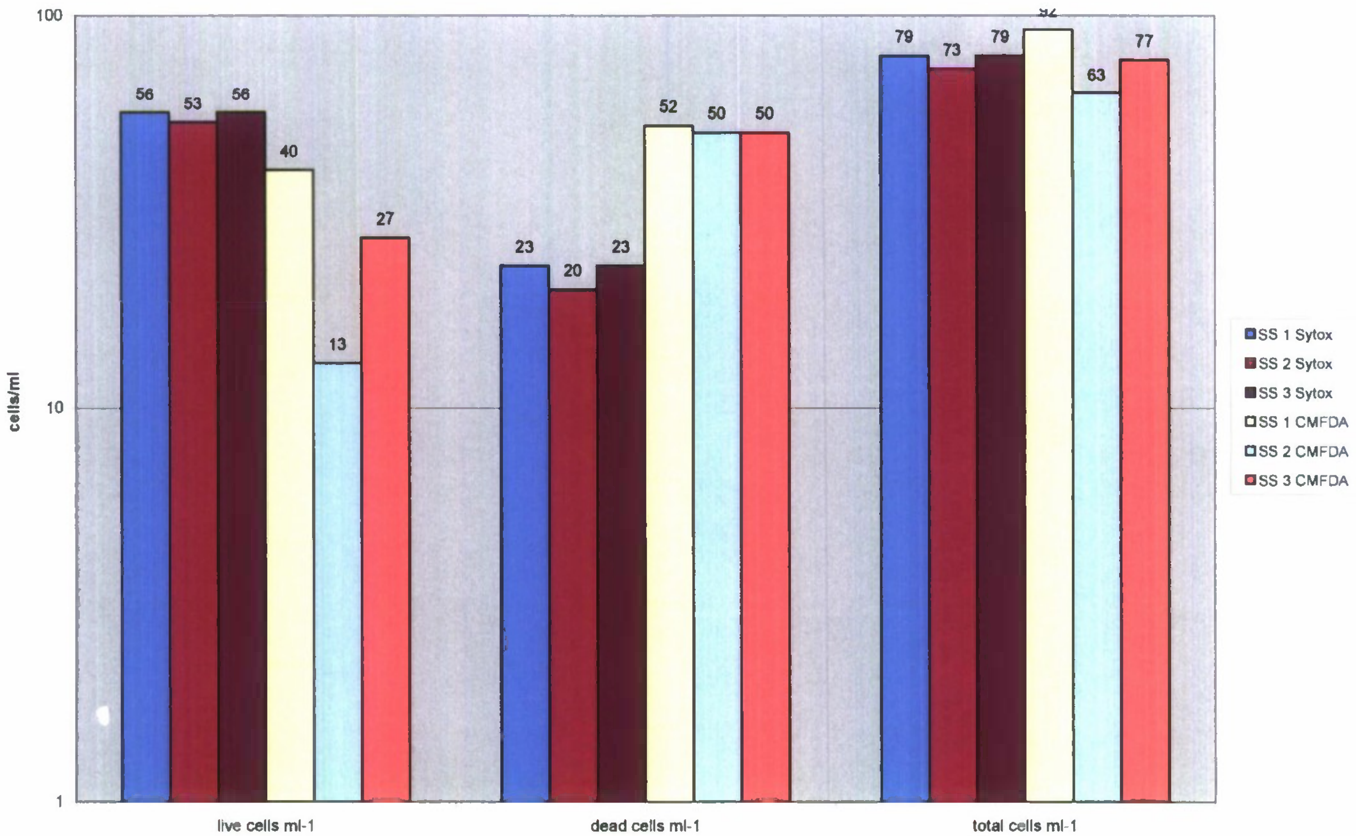

Figure 26. Filtration/microscopy counts for SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 10 AM January 13, 2008 workshop sample.

3.3.6 2:00 PM Sample from January 13, 2008 – Target Test Sample Concentration 1000 Dead: 20 Live Cells

A 300 mL sample was supplied and six 5-mL subsamples were processed for this workshop sample. Three subsamples each were enumerated for both SYTOX® Green and Cell Tracker™ Green CMFDA vital stains. Due to the high cell densities, all the cells seen in 3 sweeps across the filter counted in a modified "+"pattern (2 horizontal and 1 vertical sweeps) were tallied. As noted in the 10:00 AM sample from January 13, the Cell Tracker™ Green CMFDA -labeled cells in this sample did not seem to be as bright as those witnessed in previous sample sets. This discrepancy may be due to degradation of the DMSO used to re-suspend this probe prior to use and this is supported by the results obtained on January 14, 2008 when fresh DMSO used to re-suspend the Cell Tracker™ Green CMFDA showed consistent, brighter cell labeling.

The mean SYTOX® Green and Cell Tracker™ Green CMFDA values for live and dead cell totals in this sample were 300 live cells mL^{-1} and 580 dead cells mL^{-1} using SYTOX® Green and 174 live cells mL^{-1} and 780 dead cells mL^{-1} using Cell Tracker™ Green CMFDA (Figure 27). The dead cell concentrations were within a factor of two of the target concentration levels (but were higher than those obtained using other methods). The reported live cell concentrations were higher than the target concentrations. These results were also higher than those reported by the other workshop methods.

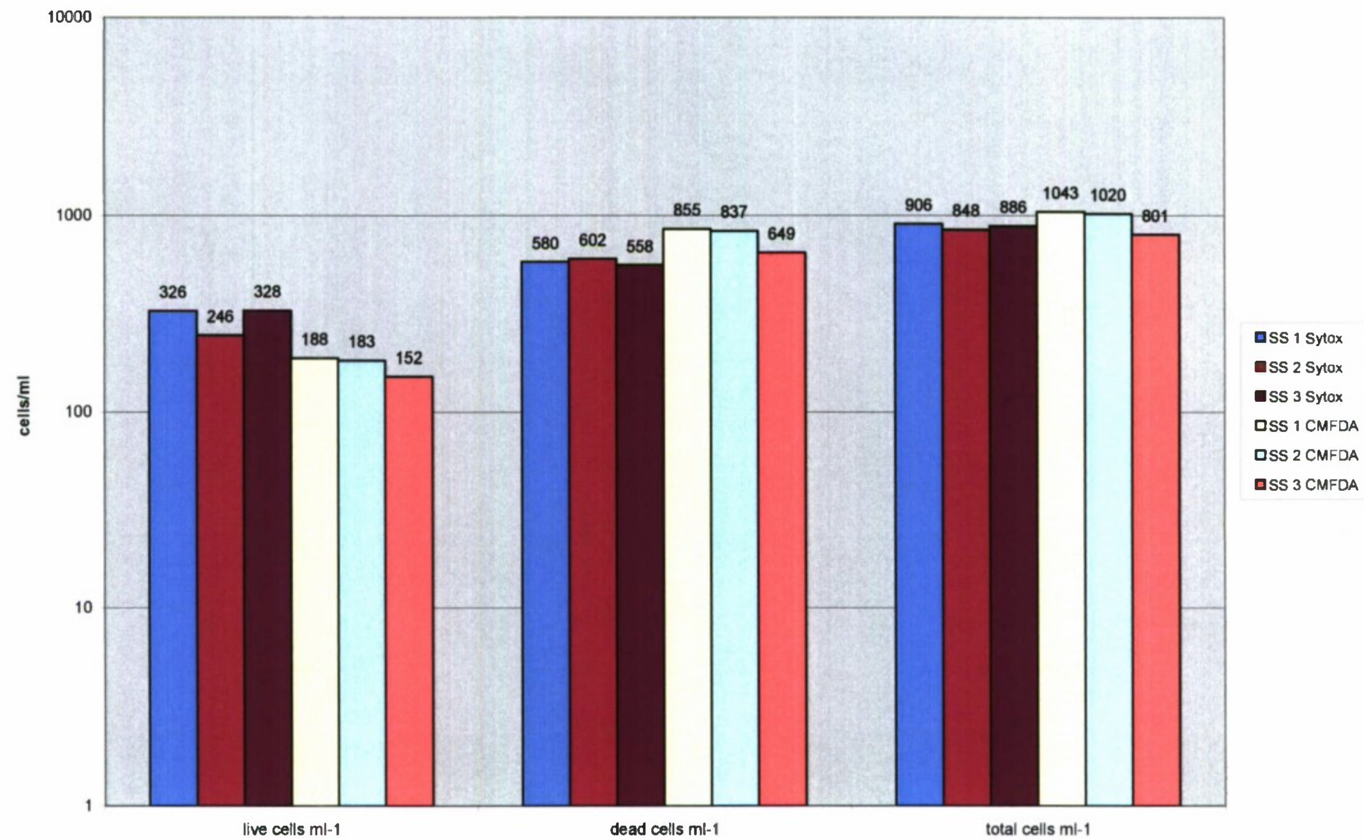

Figure 27. Filtration/microscopy counts for SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the 2 PM January 13, 2008 workshop sample.

3.3.7 Sample from January 14, 2008 – Target Test Sample Concentration 20 Live Cells With TSS and TOC Added

This sample had dissolved organic matter and suspended particulate material added at concentrations 10x greater than samples prepared for testing at the BWTTF in NRLKW. Here, the objective was to determine if these products interfered with the participants' ability to enumerate the live and dead *Tetraselmis* cells contained in this sample matrix. For the stain filtration method, the added organic and particulate matter actually enhanced visualization of the labeled and unlabeled cells, as the sample matrix provided more contrast to the Cyclopore filter membrane thus allowing for more rapid cell enumeration. The results are provided in Figure 28.

For this sample, 300 mL were supplied and three 5 mL subsamples were processed with the entire filter counted. Good agreement was found in the numbers of live and dead cells using both the Cell Tracker™ Green CMFDA and SYTOX® Green methods: mean live and dead cell totals in this sample were 35 live cells mL^{-1} and 2 dead cells mL^{-1} using Cell Tracker™ Green CMFDA and 37 live cells mL^{-1} and 7 dead cells mL^{-1} using SYTOX® Green.

The complexity of the sample had little negative impact on the ability to efficiently characterize this workshop test sample

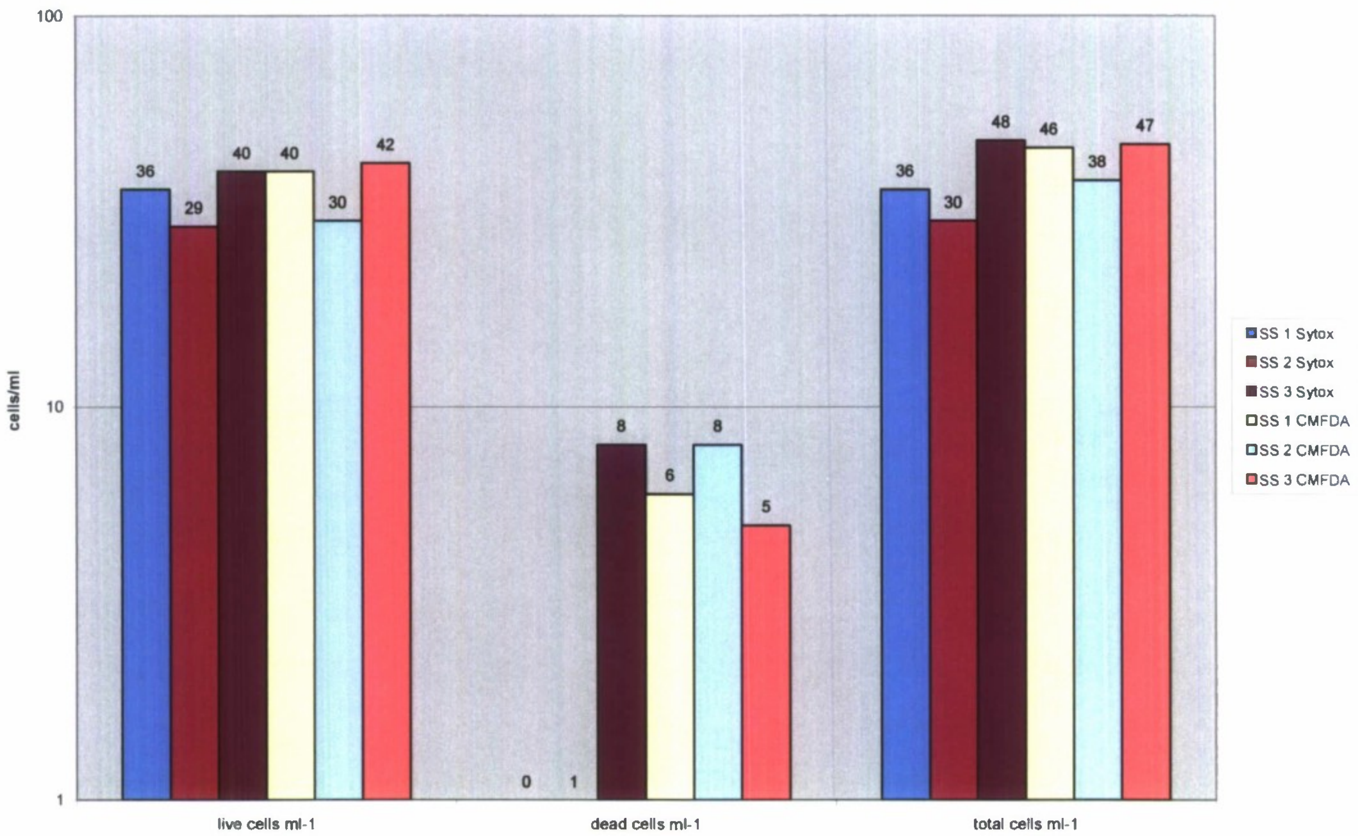

Figure 28. Filtration/microscopy counts for SYTOX® Green and Cell Tracker™ Green CMFDA stained samples for the January 14, 2008 workshop sample with added POC, TOM, and MM.

3.4 Moss Landing Marine Laboratory – Various Methods

3.4.1 10 AM Sample from January 11, 2008 – Target Test Sample Concentration 300 Dead: 30 Live Cells

Figure 29 provides the results obtained by the MLML's flow cytometer for five subsamples of this sample, which was stained using only SYTOX® Green. On average, 8 live cells mL^{-1} and 135 dead cells mL^{-1} were detected for this sample.

The results obtained with this sample are generally consistent across each of the subsamples analyzed. Additionally, the reported results are fairly consistent with the target phytoplankton concentrations (within a factor of 2) for this sample. PAM results (from MLML) from this sample were an F_V/F_M ratio value of 0.43 indicating viable cells were present in this workshop sample.

MPN values for this workshop sample were 7.8 viable cells mL^{-1}. This value is consistent with those obtained through flow cytometry as well as other workshop methods.

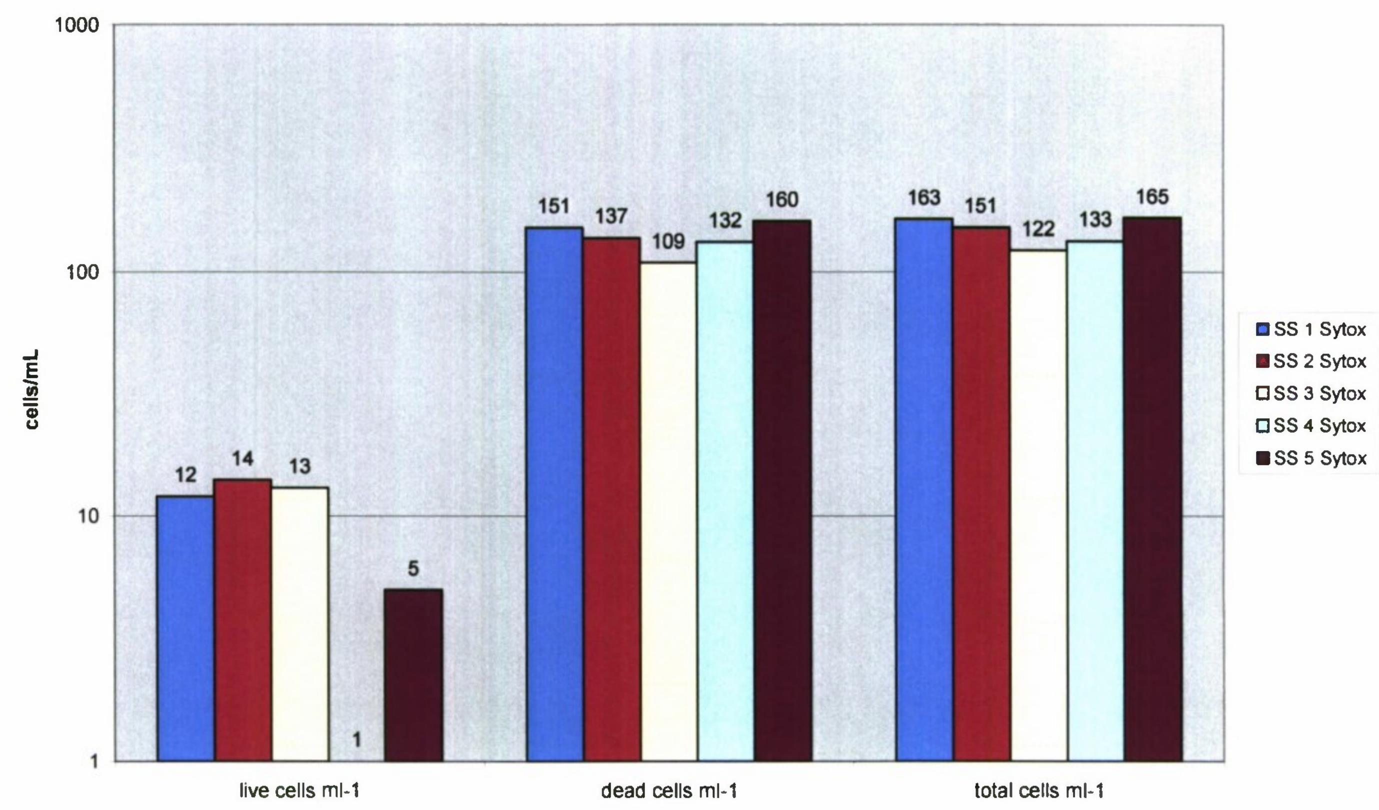

Figure 29. MLML flow cytometer data for SYTOX® stained subsamples from the 10 AM January 11, 2008 workshop sample.

3.4.2 2:00 PM Sample from January 11, 2008 – Target Test Sample Concentration 1000 Dead: 100 Live Cells

Figure 30 provides the results obtained by the MLML's flow cytometer for each of the five subsamples for this sample. In this instance, only SYTOX® Green was used as a stain. On average, 41 live cells mL^{-1} and 157 dead cells mL^{-1} were detected.

As shown in Figure 30, the results obtained with this sample are very consistent across each of the subsamples analyzed, and additionally, they are fairly consistent with the target phytoplankton concentrations (nearly within a factor of 2).

The PAM results from this sample yielded an F_V/F_M ratio value of 0.42 (for 41 live cells), indicating viable cells were present. It should be noted, however, that this ratio is inconsistent with the ratio obtained in the 10 AM sample (0.43 for 8 live organisms). All other phytoplankton enumeration methods detected approximately two times more viable cells in the 2:00 PM than in the 10 AM sample, but this difference was not reflected in the F_V/F_M ratios.

The MPN values for this sample were 8.2 viable cells mL^{-1}, a value also much closer to the 10 AM sample and much lower than that generated by all of the other workshop methods.

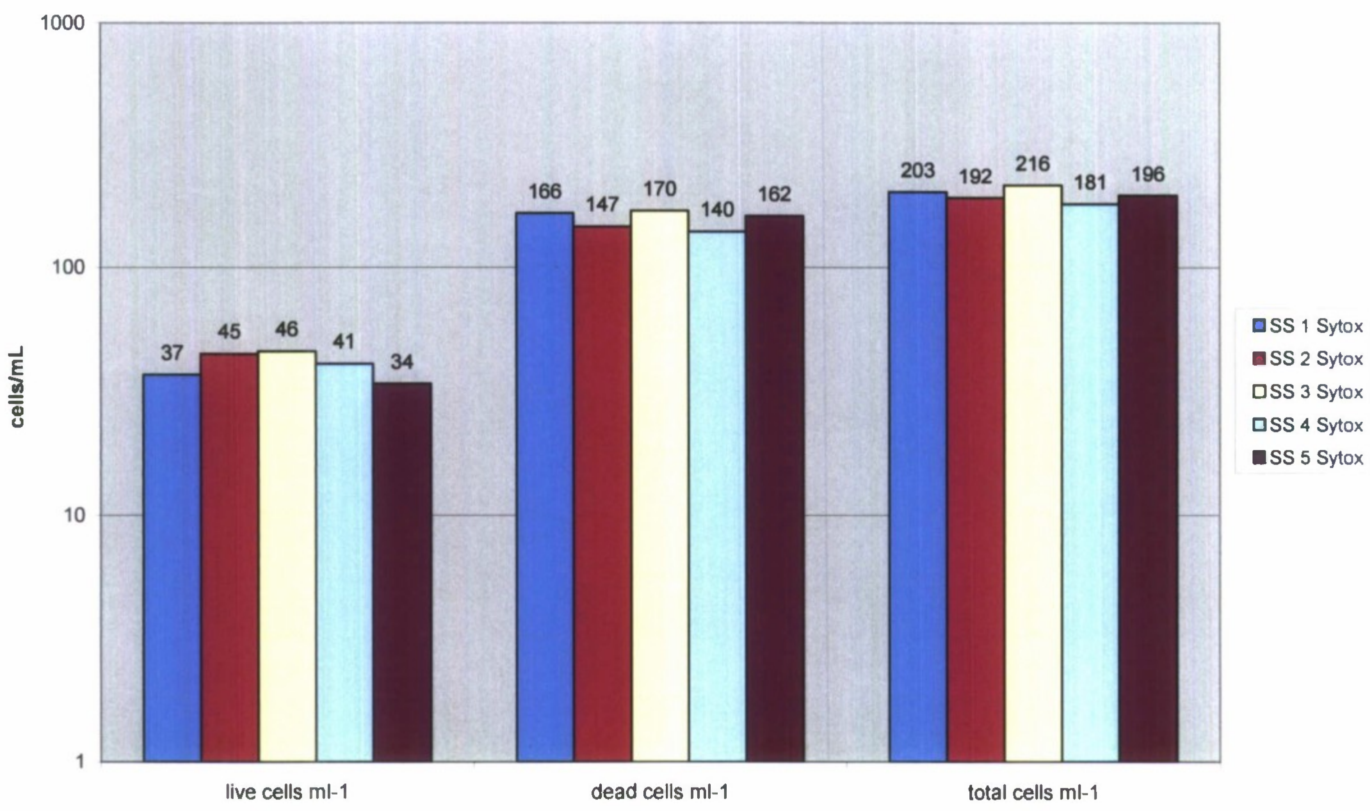

Figure 30. MLML flow cytometer data for SYTOX® Green stained subsamples from the 2 PM January 11, 2008 workshop sample.

3.4.3 10:00 AM Sample from January 12, 2008 – Target Test Sample Concentration 141 Dead: 141 Live Cells

Figure 31 provides the results obtained by the MLML's flow cytometer for the four subsamples for this phytoplankton sample. Here, only SYTOX® Green was used as a stain. On average, 36 live cells mL^{-1} and 59 dead cells mL^{-1} were detected.

As shown in Figure 31, the results obtained with this sample are very consistent across each of the subsamples analyzed. Also, the reported results are fairly consistent with the target phytoplankton concentrations (within a factor of 3) for this sample.

The PAM results from this sample showed an F_V/F_M ratio value of 0.65, indicating viable cells were present in this sample. This value is significantly higher than for previous samples.

MPN values for this workshop sample were 8.2 viable cells mL^{-1}. This value is much lower than those generated by all of the other workshop methods.

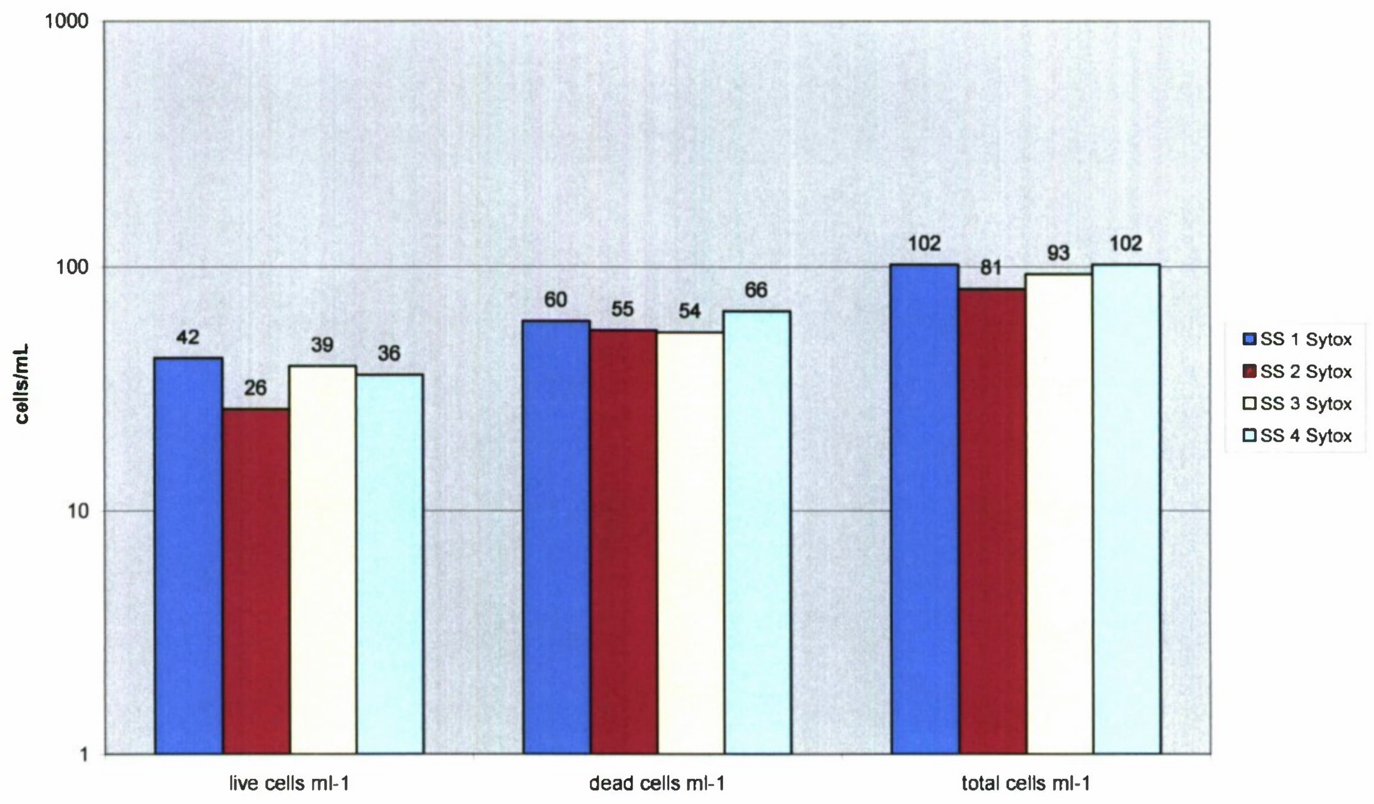

Figure 31. MLML flow cytometer data For SYTOX® Green stained subsamples from the 10 AM January 12, 2008 workshop sample.

3.4.4 10:00 AM Sample from January 13, 2008 – Target Test Sample Concentration 200 Dead: 20 Live Cells

Figure 32 provides the results obtained by MLML's flow cytometer for this sample. Both SYTOX® Green and FDA were used with this sample. Five subsamples were analyzed for each stain. On average, 37 live cells mL^{-1} and 49 dead cells mL^{-1} were detected using SYTOX® Green, and 36 live cells mL^{-1} and 69 dead cells mL^{-1} were detected using FDA.

As shown in Figure 32, the results obtained with this sample are very consistent across each of the subsamples analyzed as well as for both of the vital stains utilized. The data are within a factor of six of the target phytoplankton concentrations for this sample.

Here, PAM results showed an F_V/F_M ratio value of 0.53, indicating viable cells were present in the sample.

No MPN values were generated for this sample.

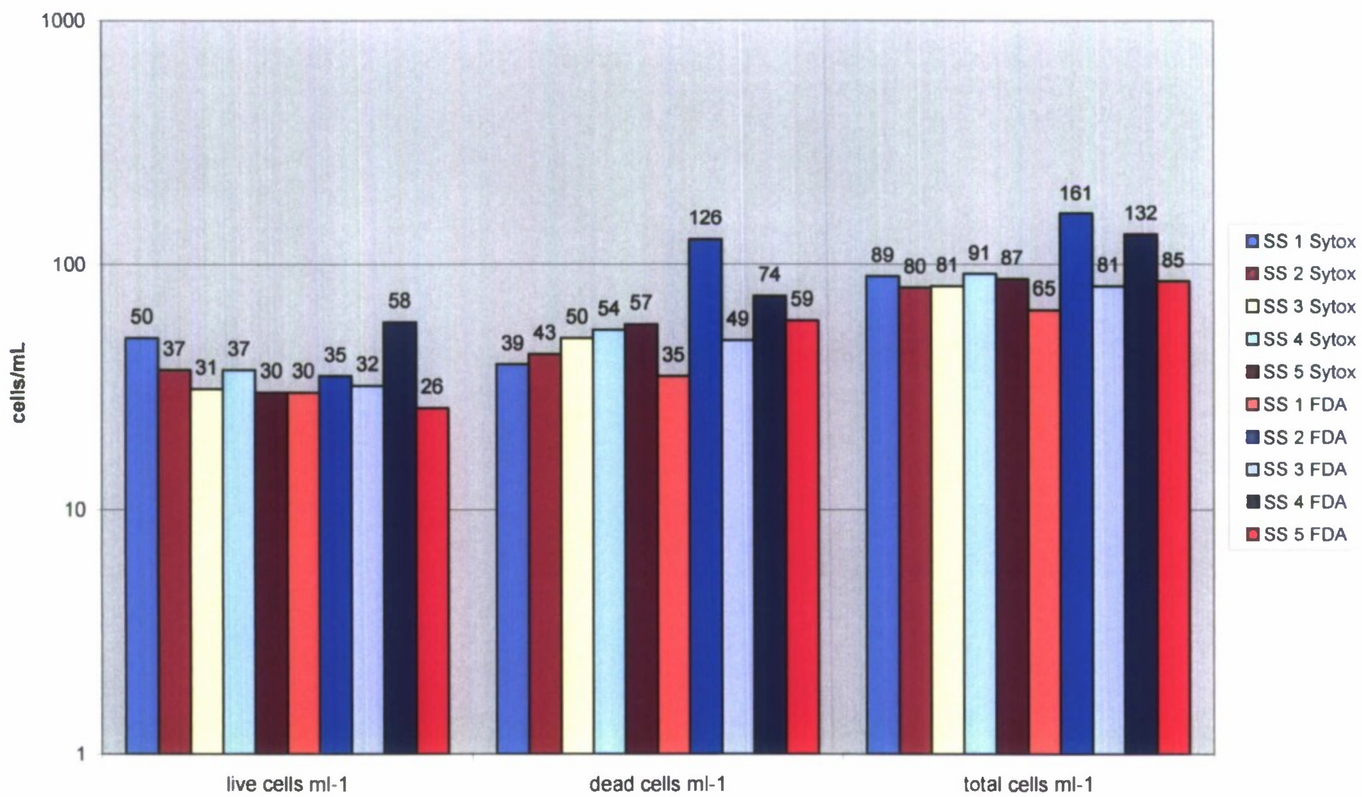

Figure 32. MLML flow cytometer data for SYTOX® and FDA stained subsamples from the 10 AM January 13, 2008 workshop sample.

3.4.5 2:00 PM Sample from January 13, 2008 – Target Test Sample Concentration 1000 Dead: 20 Live Cells

Figure 33 provides the results obtained by the MLML's flow cytometer for the five subsamples. Here, both SYTOX® Green and FDA were used. On average, 96 live cells mL^{-1} and 318 dead cells mL^{-1} were detected using SYTOX® Green, and 90 live cells mL^{-1} and 336 dead cells mL^{-1} were detected using FDA.

As shown in Figure 33, the results obtained with this sample are very consistent across each of the subsamples analyzed as well as for both of the vital stains utilized. The reported results are also fairly consistent with the target phytoplankton concentrations for this workshop test sample.

The PAM results were an F_V/F_M ratio value of 0.28 indicating that viable cells were present. This ratio is, however, inconsistent (it is low) with the results obtained for the number of viable cells using flow cytometry or the other workshop methods. Compare also to the 10:00 AM sample which also had a target value of 20 living cells and a ratio of 0.53.

No MPN values were generated for this sample.

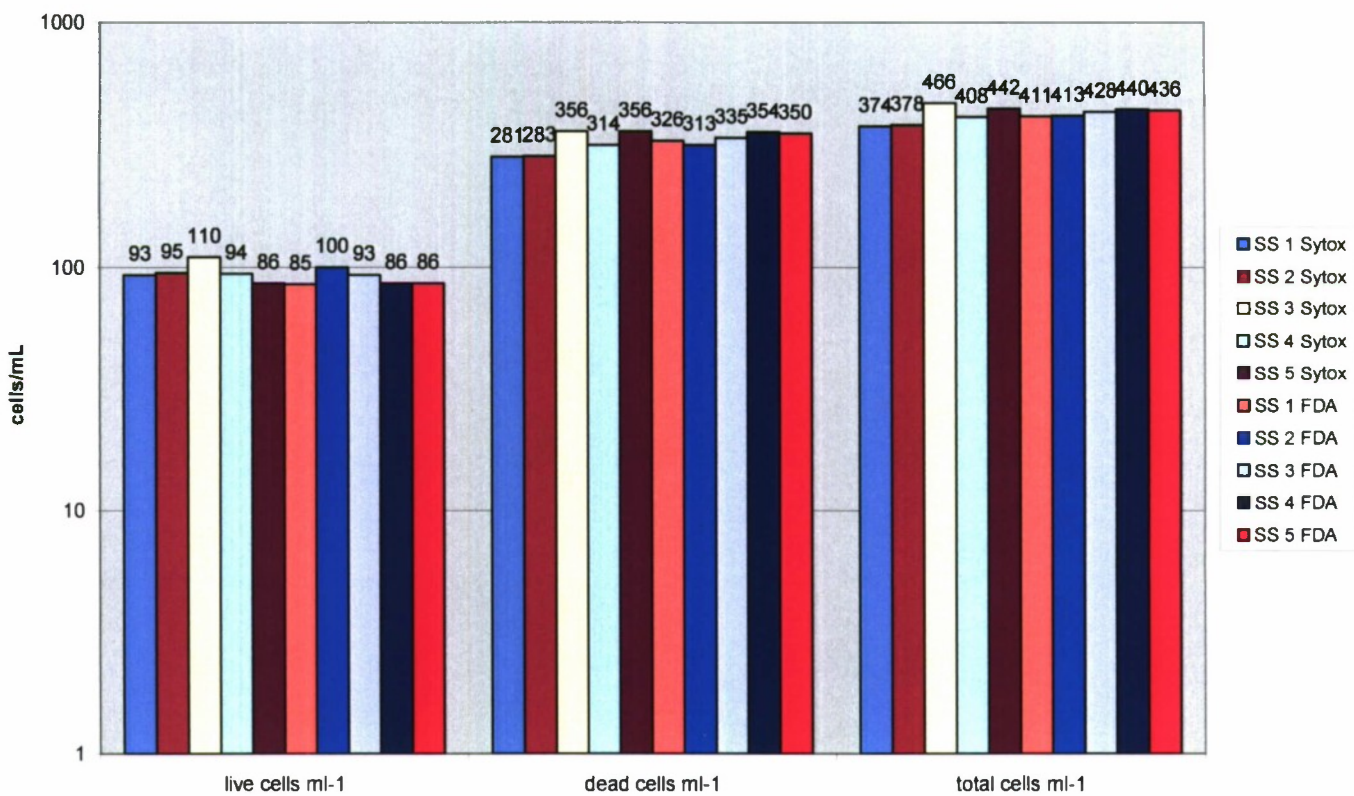

Figure 33. MLML flow cytometer data for SYTOX® and FDA stained subsamples from the 2 PM January 13, 2008 workshop sample.

3.4.6 Sample from January 14, 2008 – Target Test Sample Concentration 20 Live Cells with TSS and TOC Added

Figure 34 provides the results obtained by the MLML's flow cytometer for this sample; viability of five subsamples each was analyzed using SYTOX® Green and FDA stains. On average, 39 live cells mL^{-1} and 18 dead cells mL^{-1} were detected using SYTOX® Green, and 30 live cells mL^{-1} and 9 dead cells mL^{-1} were detected using FDA.

The results from this sample were very consistent across each of the subsamples. MLML researchers thought their dead cell counts were more reliable with the five FDA vital stain subsamples since SYTOX® Green stained the test dust, which made it difficult to detect dead cells. (Note the higher counts with SYTOX® Green stain.) The live cell counts are very consistent with both of the vital stains utilized. Finally, the results are fairly consistent with the target phytoplankton concentrations for this workshop test sample.

PAM results from this sample were an F_V/F_M ratio value of 0.33 indicating that viable cells were present in this sample.

MPN values showed 12 viable cells mL^{-1}. This value is consistent with the flow cytometry as well as other workshop methods.

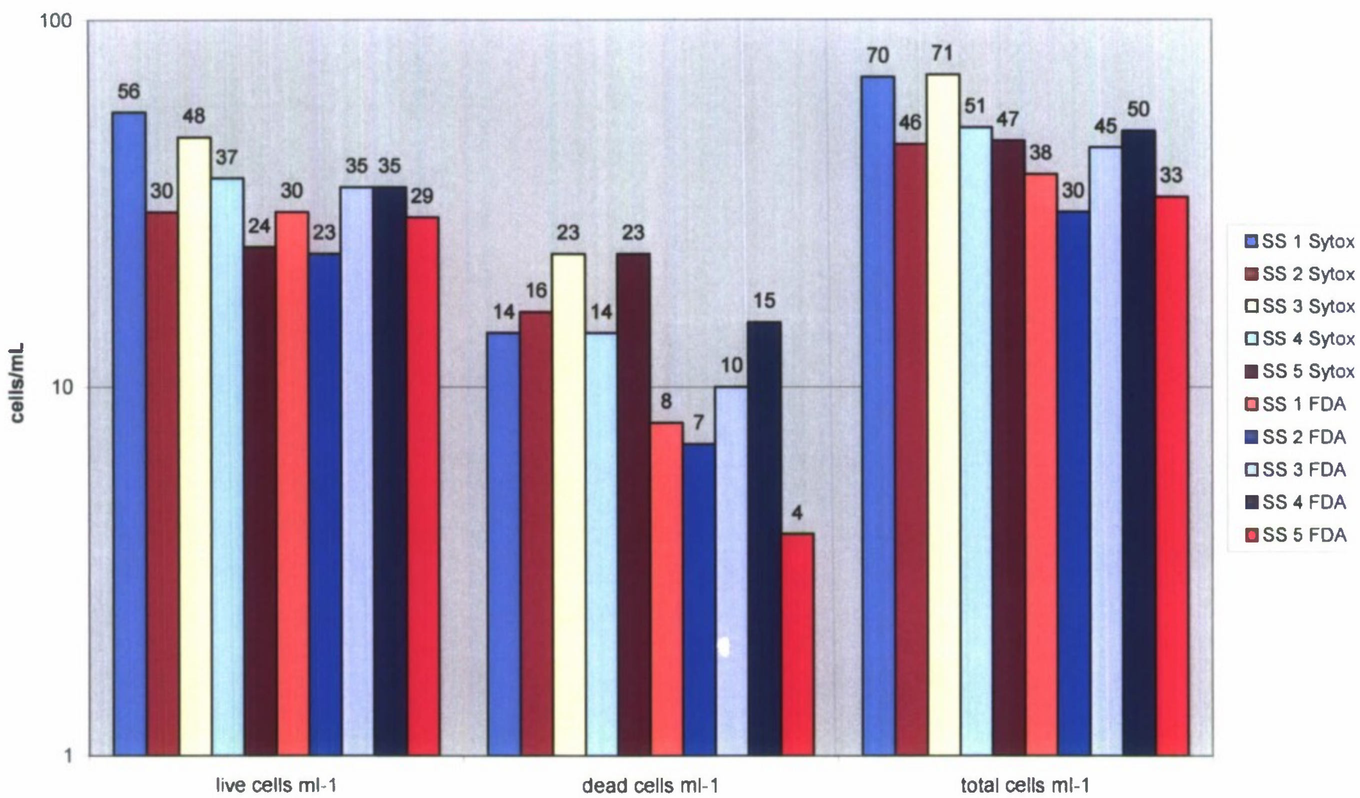

Figure 34. MLML flow cytometer data for SYTOX® Green and FDA stained subsamples from the January 14, 2008 workshop sample with added POC, TOM, and MM.

3.5 NRLKW/Fluid Imaging Technologies –FlowCAM®

The methods described in section 2.5 of this report were used to analyze the majority of the samples described in this section of the report. Where there was a deviation in these methods, it is noted in the narrative.

3.5.1 10 AM Sample from January 11, 2008 – Target Test Sample Concentration 300 Dead: 30 Live Cells

The results obtained using the FlowCAM® from this test sample are provided in Figure 35. Two 1-mL subsamples were analyzed from this workshop test sample, and the results showed means of 21 viable phytoplankton cells mL^{-1} and 150 non-viable cells mL^{-1}. Detected phytoplankton cells of unknown viability were 23 ± 15 mL^{-1}.

The live cell counts for this sample are consistent across the subsamples analyzed. Both the live and dead counts were within a factor of 2 of the target phytoplankton concentrations for this workshop test sample.

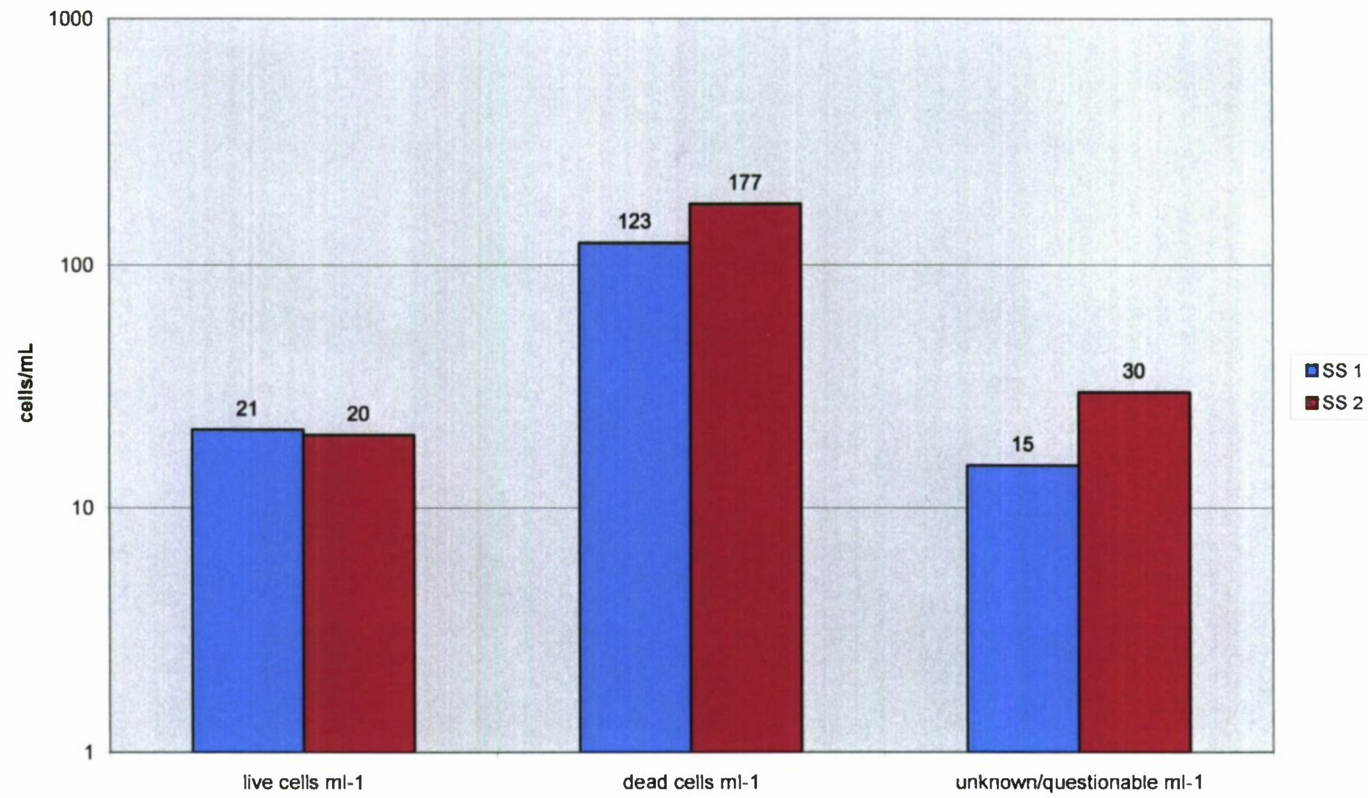

Figure 35. FlowCAM® counts for the 10 AM January 11, 2008 workshop sample.

3.5.2 2:00 PM Sample from January 11, 2008 – Target Test Sample Concentration 1000 Dead: 100 Live Cells

The results obtained using the FlowCAM® on three 1-mL subsamples for this test sample are provided in Figure 36. The first two 1-mL subsamples were diluted by a 5:1 ratio using artificial seawater prior to their introduction to the FlowCAM®. The last 1-mL subsample was introduced into the FlowCAM® undiluted. Please note that the undiluted sample is reported as replicate 1 (blue) in Figure 36.

The mean results obtained on this sample were 42 viable phytoplankton cells $mL^{-1}$and 258 non-viable phytoplankton cells mL^{-1}. Detected phytoplankton cells of unknown viability were 30 ± 20 mL^{-1}.

The number of viable (live) phytoplankton mL^{-1} detected in this workshop test sample was consistent across each of the three subsamples including the diluted subsample.

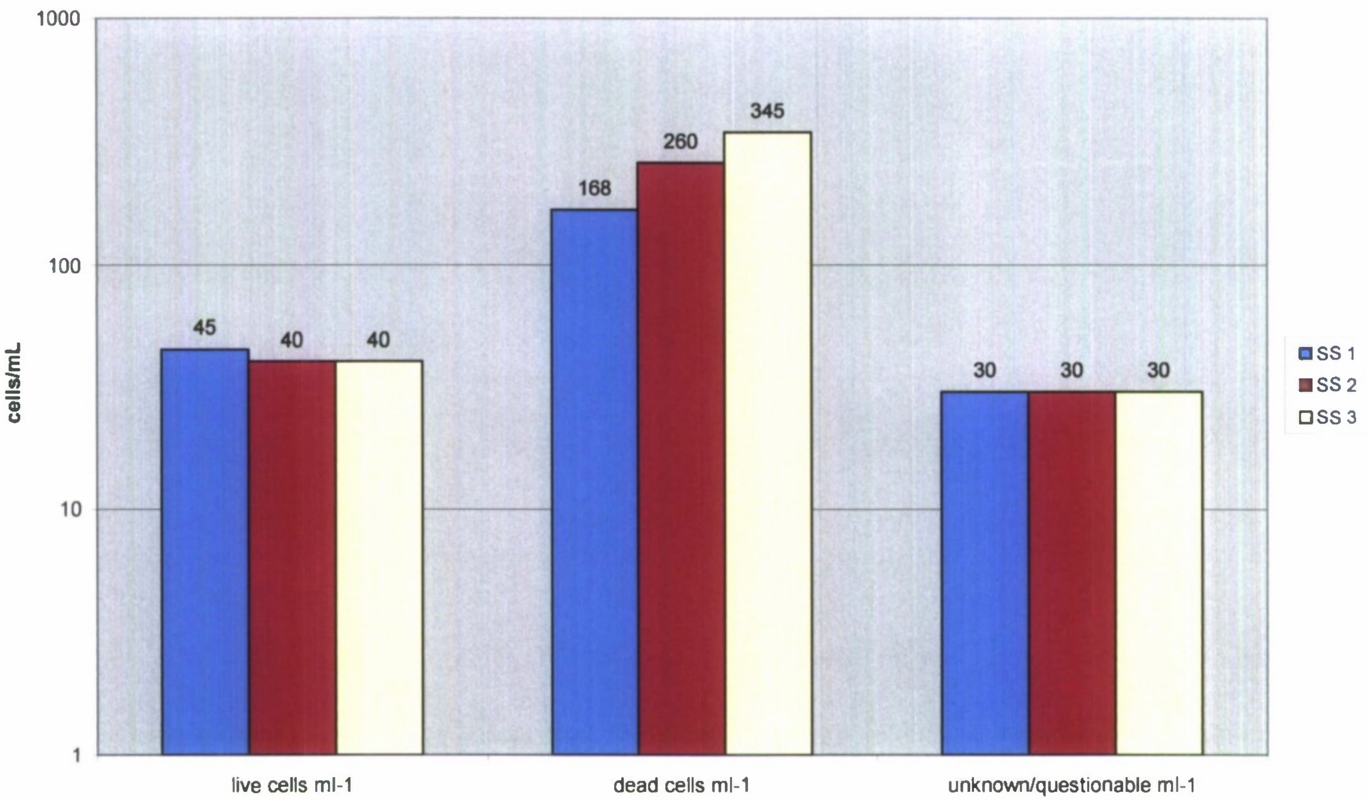

Figure 36. FlowCAM® counts for the 2 PM January 11, 2008 workshop sample.

3.5.3 10:00 AM Sample from January 12, 2008 – Target Test Sample Concentration 141 Dead: 141 Live Cells

The results obtained using the FlowCAM® from this test sample are provided in Figure 37. Five 1-mL subsamples were analyzed. A small leak was observed at the top of the flow cell during the processing of the third subsample. A new flow cell was installed into the FlowCAM® following the processing of this subsample, and the system's optical alignment and focus were optimized. This flow cell was used in the FlowCAM® to process the remaining workshop test samples. The mean results from this sample were 59

viable phytoplankton cells mL^{-1} and 18 non-viable cells mL^{-1}. The number of detected phytoplankton cells of unknown viability was 68 ± 41 mL^{-1} (Figure 37).

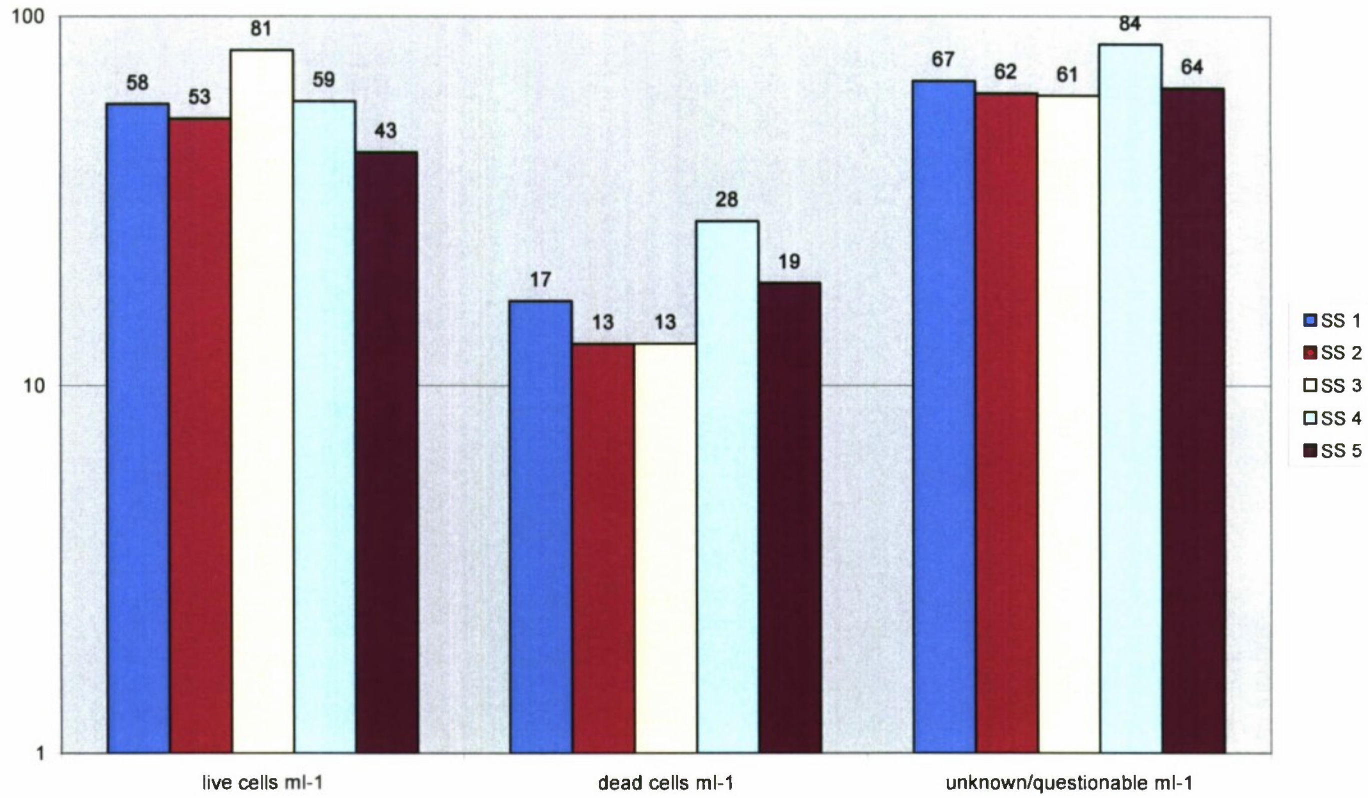

Figure 37. FlowCAM® counts for the 10 AM January 12, 2008 workshop sample.

The number of viable phytoplankton detected in this workshop test sample was fairly consistent across each of the five subsamples. As was the case with previous workshop samples, the results reported are approximately within a factor of two of the target viable cell concentrations for this workshop test sample.

The number of non-viable phytoplankton detected in this workshop test sample was very consistent across each of the five subsamples. These results are, however, much lower than both the target concentration levels for this test sample as well as the results reported by the other workshop participants.

The unknown/questionable phytoplankton that were detected in this workshop test sample was also fairly consistent across the five subsamples analyzed. It is unclear why there were such a large number of phytoplankton cells detected by the FlowCAM® that showed no signs of either chlorophyll *a* or SYTOX® Green fluorescence for this test sample. There is also the potential for large errors in these results as they were obtained exclusively through a manual analysis of the particle images collected using the FlowCAM®, and these images are spatial resolution limited due to the 10X objective configuration that was used in the FlowCAM® to support the workshop.

It should be noted that the total number of cells reported (viable, non-viable and unknown) for this sample was very consistent with the results obtained by the other workshop participants. Further, if the majority of the unknown/questionable cells that were detected were not viable, then the overall results obtained in this sample would be fully consistent with those reported by the other workshop participants.

3.5.4 2:00 PM Sample from January 12, 2008 – Target Test Sample Concentration 20 Dead: 20 Live Cells

The results obtained using the FlowCAM® for this sample are provided in Figure 38. Five 1-mL subsamples were analyzed. The mean results from this sample were 12 viable phytoplankton cells mL^{-1} and 12 non-viable cells mL^{-1}. Detected phytoplankton cells of unknown viability were 26 ± 24 mL^{-1}.

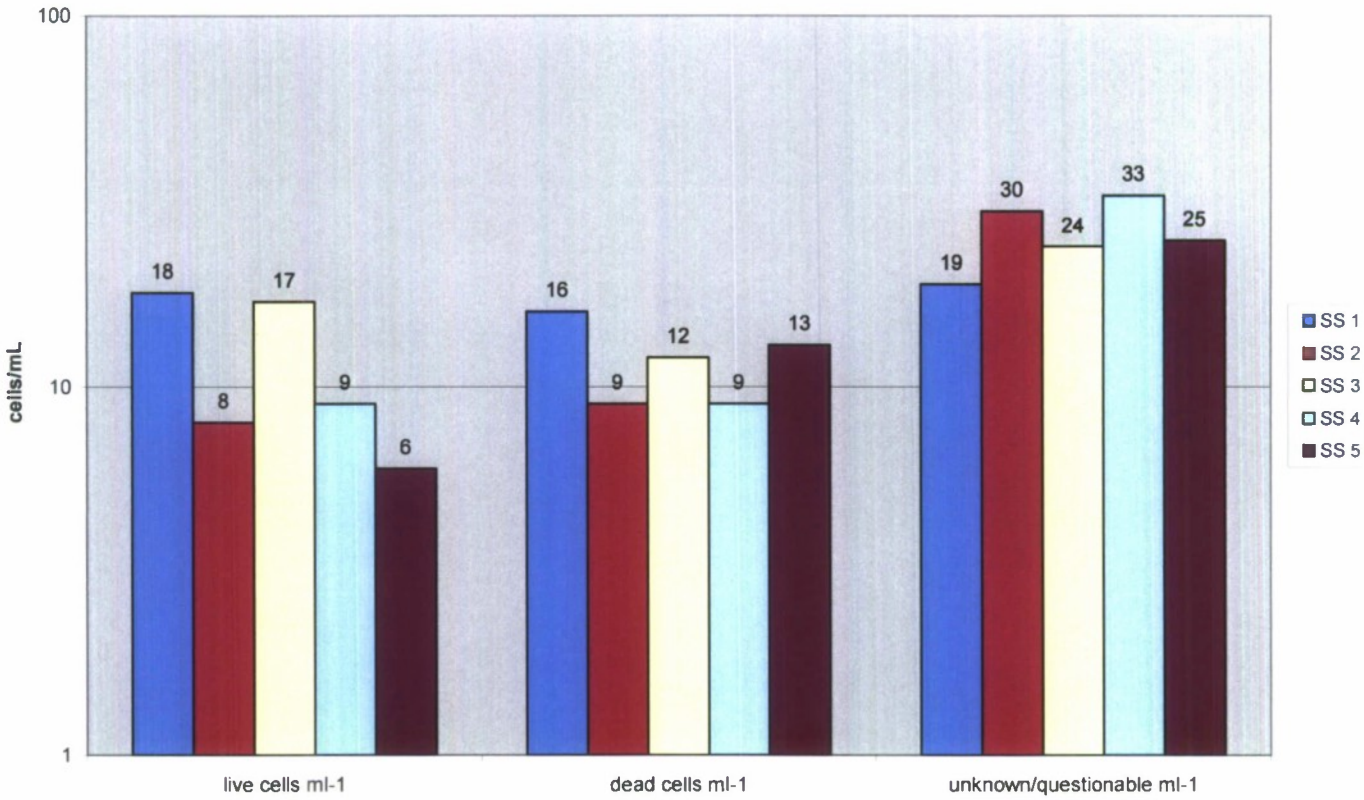

Figure 38. FlowCAM® counts for the 2 PM January 12, 2008 workshop sample.

The number of viable phytoplankton cells detected in this sample was fairly consistent across each of the five subsamples analyzed. As was the case with previous workshop samples, the results were within a factor of two of the target viable cell concentrations for this sample.

The number of non-viable phytoplankton that were detected was also very consistent across each of the five subsamples analyzed. These results are also within a factor of two of the target concentration levels for this test sample.

The number of unknown/questionable cells detected resulted in the total cell mL^{-1} for this test sample being higher than that reported by the other workshop participants. Nonetheless, it is important to note the high degree of uncertainty in these numbers.

3.5.5 10:00 AM Sample from January 13, 2008 – Target Test Sample Concentration 200 Dead: 20 Live Cells

The results obtained using the FlowCAM® for this test sample are provided in Figure 39. From the five 1-mL subsamples analyzed, the means were 21 viable phytoplankton cells mL^{-1} and 26 non-viable cells mL^{-1}. The number of detected phytoplankton cells of unknown viability was 22 ± 16 mL^{-1}.

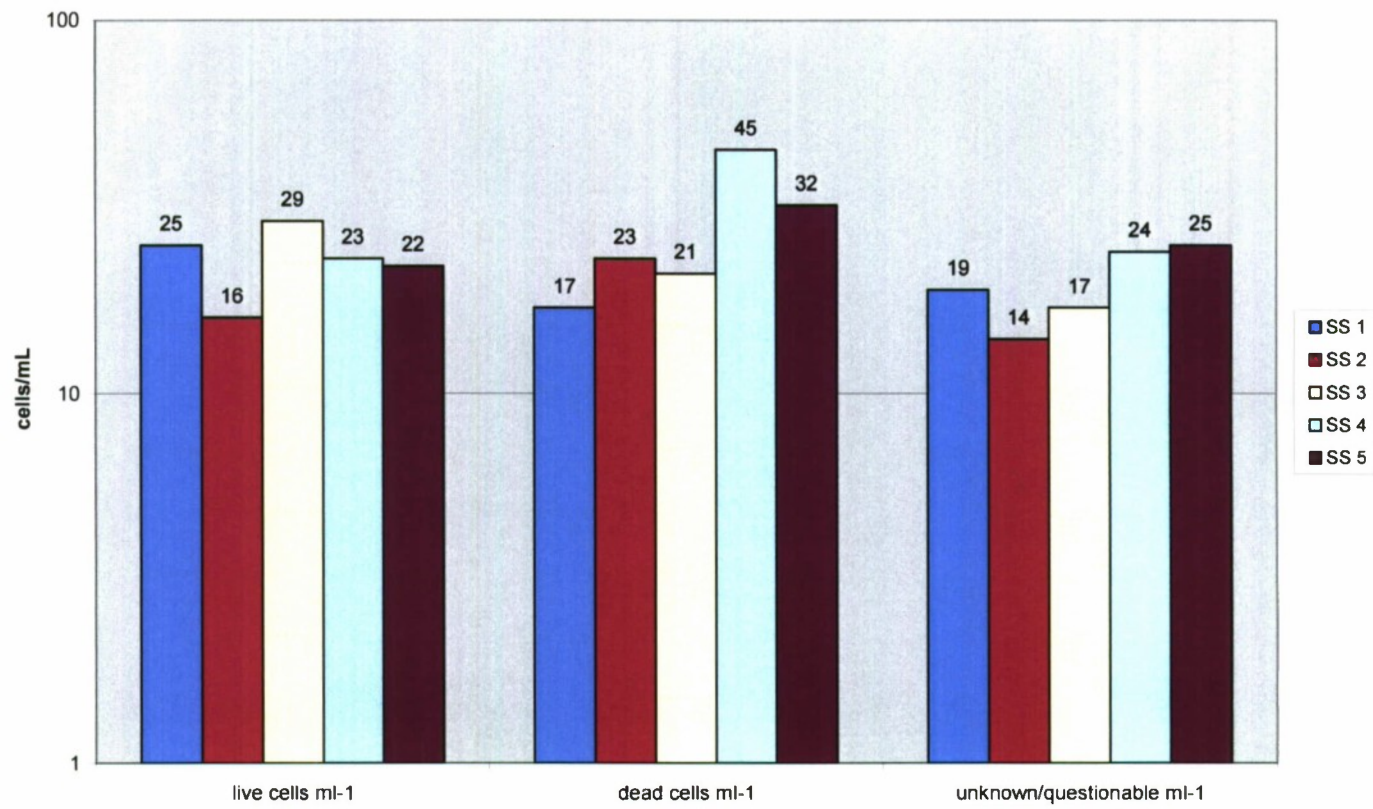

Figure 39. FlowCAM® counts for the 10 AM January 13, 2008 workshop sample.

The number of viable phytoplankton detected in this sample was very consistent across each of the five subsamples analyzed. As was the case with previous workshop samples, the results reported are within a factor of two of the target viable cell concentrations for this workshop test sample.

The number of non-viable phytoplankton and the total number of phytoplankton cells (live + dead + unknown) detected was consistent across the first three sample replicates analyzed. The reported results for all five subsamples are significantly less than the target concentrations for this test sample. On the other hand, the results reported by each of the other workshop participants were also significantly lower than the target concentration for dead cells.

3.5.6 2:00 PM Sample from January 13, 2008 – Target Test Sample Concentration 1000 Dead: 20 Live Cells

The results obtained using the FlowCAM® from this test sample are provided in Figure 40. As before, five 1 mL subsamples were analyzed. The mean results from this sample were 78 viable phytoplankton cells mL^{-1} and 262 non-viable cells mL^{-1}. The number of detected phytoplankton cells of unknown viability was $55 \pm 16\ mL^{-1}$.

The number of viable phytoplankton detected in this test sample was very consistent across each of the five subsamples analyzed although the results were higher than the target concentration. Even though results were less consistent than in other workshop test samples, the majority of the workshop participants also reported viable cell concentrations that were much greater than the target concentration levels for this test sample.

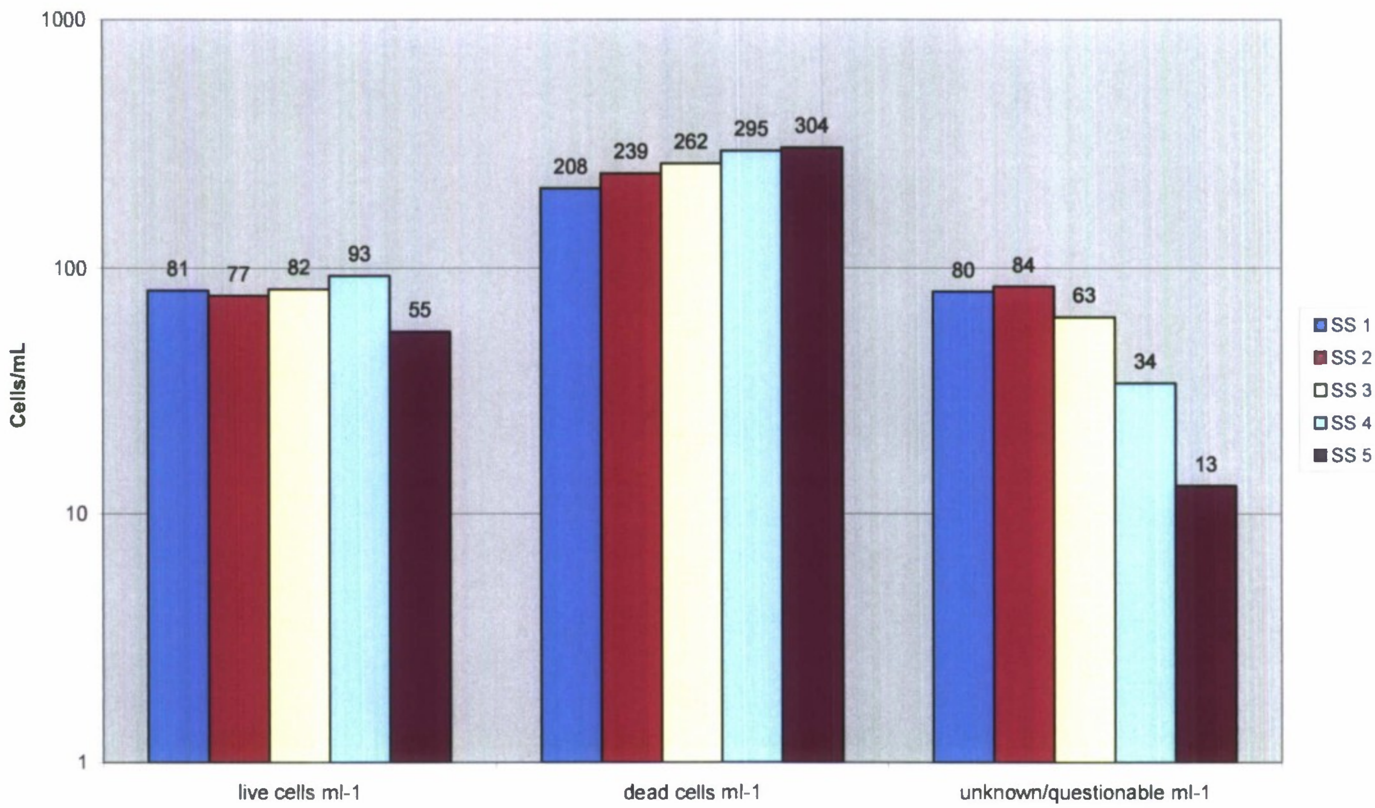

Figure 40. FlowCAM® counts for the 2 PM January 13, 2008 workshop sample.

The number of non-viable phytoplankton detected in this workshop test sample showed a steady increase as the subsamples were processed. The non-viable phytoplankton results for this workshop sample were a little more than a factor of three less than the target concentration levels for this test sample.

The total number of phytoplankton cells (live + dead + unknown) detected in this workshop test sample was consistent across the five subsamples analyzed. The results are much less than the target concentrations for this test sample.

3.5.7 Sample from January 14, 2008 – Target Test Sample Concentration 20 Live Cells with TSS and TOC Added

As mentioned is Section 2.5.2 of this report, the methods used to introduce, process, and analyze the data from the workshop test sample with added total suspended solids and total organic carbon had to be modified because of the high concentrations of non-neutrally buoyant test dust that had taken on the SYTOX® Green stain. The revised method allowed only the total number of phytoplankton cells to be reported for this sample. Because many phytoplankton cells do not produce a chlorophyll *a* fluorescence signal that can be measured by the FlowCAM®, the results reported for this test sample are expected to be lower than the actual number of cells in the test sample. It is also anticipated that at least 50% of the cells reported are viable (based on the typical number of cells that were detected with chlorophyll *a* fluorescence on the other test runs). Nonetheless, there is no way to validate this conclusion with the data that were collected on this workshop test sample.

Four 5 mL subsamples diluted with artificial seawater by a 5:1 ratio before being introduced into the FlowCAM® were analyzed. The results for these four subsamples were:

Subsample 1: 48 phytoplankton cells mL^{-1}

Subsample 2: 33 phytoplankton cells mL^{-1}

Subsample 3: 43 phytoplankton cells mL^{-1}

Subsample 4: 25 phytoplankton cells mL^{-1}

These data are consistent across the four subsamples. These results are additionally consistent with the total cell concentrations reported by the other workshop participants.

3.6 Results Comparison across Methods

This report section provides tables and figures that allow the results for each of the methods to be compared to each another for the seven workshop samples. Table 4 provides a quick review of the mean live cell concentrations for each method for each workshop sample analyzed. As indicated here and in the previous discussions of the individual method results, the outcomes of the individual methods were quite similar to each other and generally fell within a factor of two compared to the target concentration for live organisms.

Table 4. Summary of results across methods for live phytoplankton. Note that a green background in the table indicates that the measurement was within a factor of two of the target concentration and that an orange background indicates the measurement was within a factor of four of the target concentration.

Sample #	Target Live Cell Concentration (cells/mL-1)	NRL Microscopy (Sytox®) (cells/mL-1)	WHOI Microscopy (Sytox®) (cells/mL-1)	WHOI Microscopy (CMFDA) (cells/mL-1)	MLML Flow Cytometry (SYTOX®) (cells/mL-1)	MLML Flow Cytometry (FDA) (cells/mL-1)	NRL FlowCAM® (cells/mL-1)
1	30	26	30	22	8	NA	21
2	100	53	181	193	41	NA	42
3	141	32	40	100	36	NA	59
4	20	19	15	14	NA	NA	12
5	20	23	55	27	37	36	21
6	20	17	300	174	96	90	78
7	20	53	35	27	39	30	37

To generate the figures provided in this sub-section, the subsample results for each method were averaged. In addition, an Analysis of Variance (ANOVA) was applied to each method separately for each of the workshop samples to check for differences among methods. These analyses show that there are statistically significant differences among methods for some of the workshop samples. That is, the differences between methods are large relative to the variability within sub-samples analyzed by a given method. These differences, however, are not consistent across tests, so it is not clear that the error can be specifically attributed to the methods especially when other potential sources of variability are considered. For example, it is not certain that each group received equivalent subsamples of the prepared stocks. Further, these measurement techniques are complicated, with many opportunities for mistakes to be made. The differences among methods are not particularly large in practical terms. Most of the ratios of method averages are within a factor of two, so any of the workshop methods should produce reasonable results.

To check for differences among methods, ANOVA was applied to each test separately. The ANOVA results are provided in Tables C-1 through C-7 (Appendix C). Since the ANOVA assumptions are not met exactly, the results (p-values, confidence intervals) are not exact, but they should provide a good indication of where there are real differences among the methods. The confidence intervals in the tables were derived using the pooled standard deviations ($s = MSE^{1/2}$). There are two reasons for this. First, the analysis method assumes equal variability for each factor level. If that assumption is correct, it makes sense to combine all available information to obtain the best possible estimate of that variability. Second, there is only a small amount of data available for any given test and method. Confidence intervals based on such limited information tend to be so wide they are not of much use.

The discussions of results provided for each of the individual workshop samples are primarily based on the ANOVA analyses. These analyses are summarized in the tables provided in Appendix C.

3.6.1 Sample # 1 - 10 AM Sample from January 11, 2008 – Target Test Sample Concentration 300 Dead: 30 Live Cells

Figure 41 provides a summary of the results reported by each team using various workshop methods for the 10 AM sample, January 11. Note that as indicated in the figure, MLML utilized only SYTOX® Green stain when analyzing this workshop sample using their Flow Cytometer.

The dead and total cell count results are fairly comparable across the five measurement methods used for this test. There is one significant difference in the live results. The average result for flow cytometry with SYTOX® Green stain (FCM S in Table C-1 of Appendix C) is lower than those for the other methods. The results from the flow cytometry method were more variable than would be expected from a Poisson model, and there may have been problems with the measurements. As indicated in the figure, all other methods reported results that were within a factor of two each other for the live, dead, and total phytoplankton concentration levels.

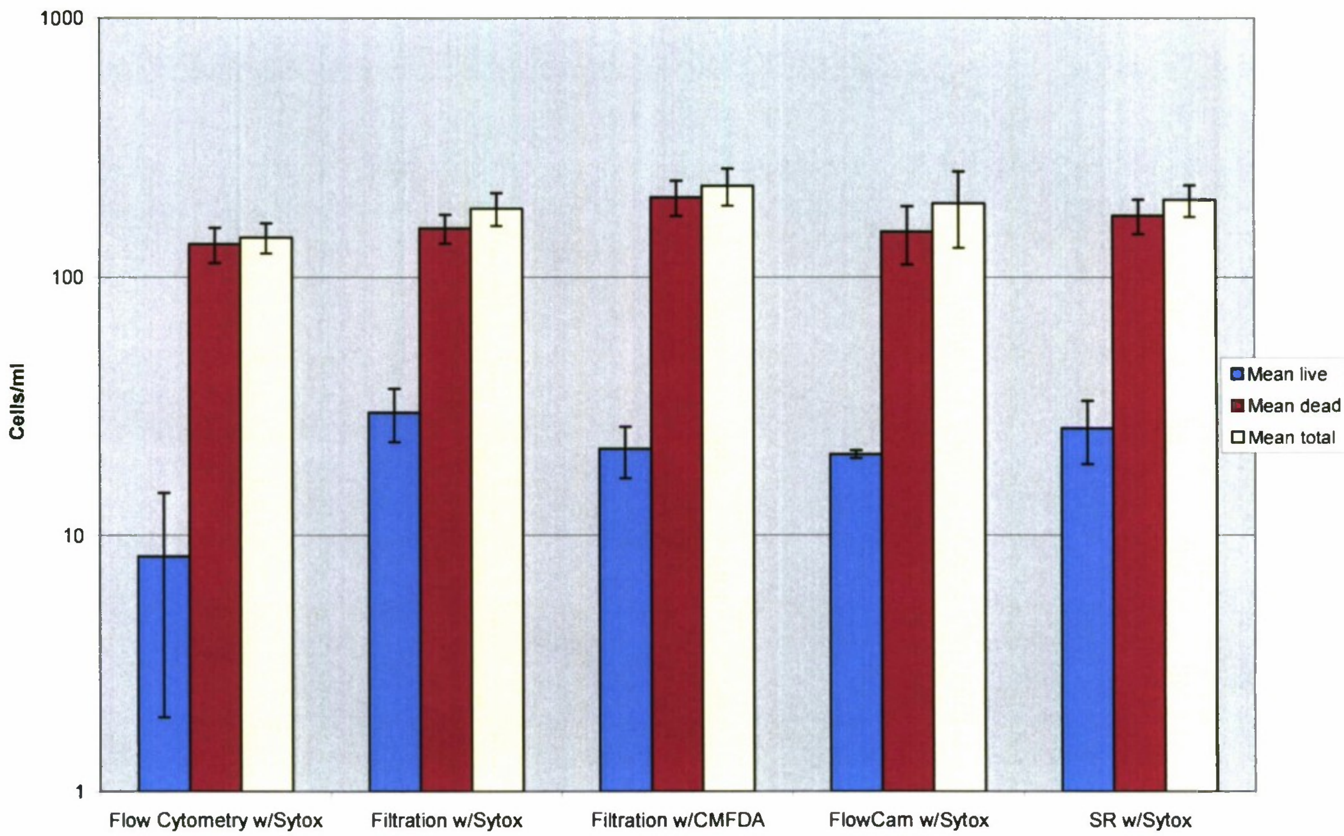

Figure 41. Comparison of different workshop methods for Sample # 1 – the 10 AM January 11, 2008 workshop sample.

3.6.2 Sample # 2 - 2:00 PM Sample from January 11, 2008 – Target Test Sample Concentration 1000 Dead: 100 Live Cells

Figure 42 provides a summary of the results reported by the different teams using the various workshop methods for the Sample 2 (2:00 PM January 11). Note that MLML only utilized SYTOX® Green stain when analyzing this workshop sample using their Flow Cytometer.

The p-values generated from the ANOVA analysis in Table C-2 for the dead and total results (0.043 and 0.019, respectively) for this test, while less than 0.05, were not small enough to provide convincing evidence of method differences. This is indicated by the Analysis of Variance assumptions not being met precisely, which can result in the calculated p-values not being exact. Furthermore, apparently significant results are obtained solely by chance on occasion. However, these results do meet the usual standard for statistical significance and as such these data indicate differences in the measurement methods.

The results obtained for the live cells however, clearly meet the criterion that indicates a significant difference in the measurement methods. ($p < .001$). The data provided in the figure clearly shows that the filtration data provides higher live cell counts than the other methods. This difference drives the p value to the lower level indicated in Table 7 It should be noted however, that all of the methods reported results that were within a factor of two each other for the live, dead, and total phytoplankton concentration levels.

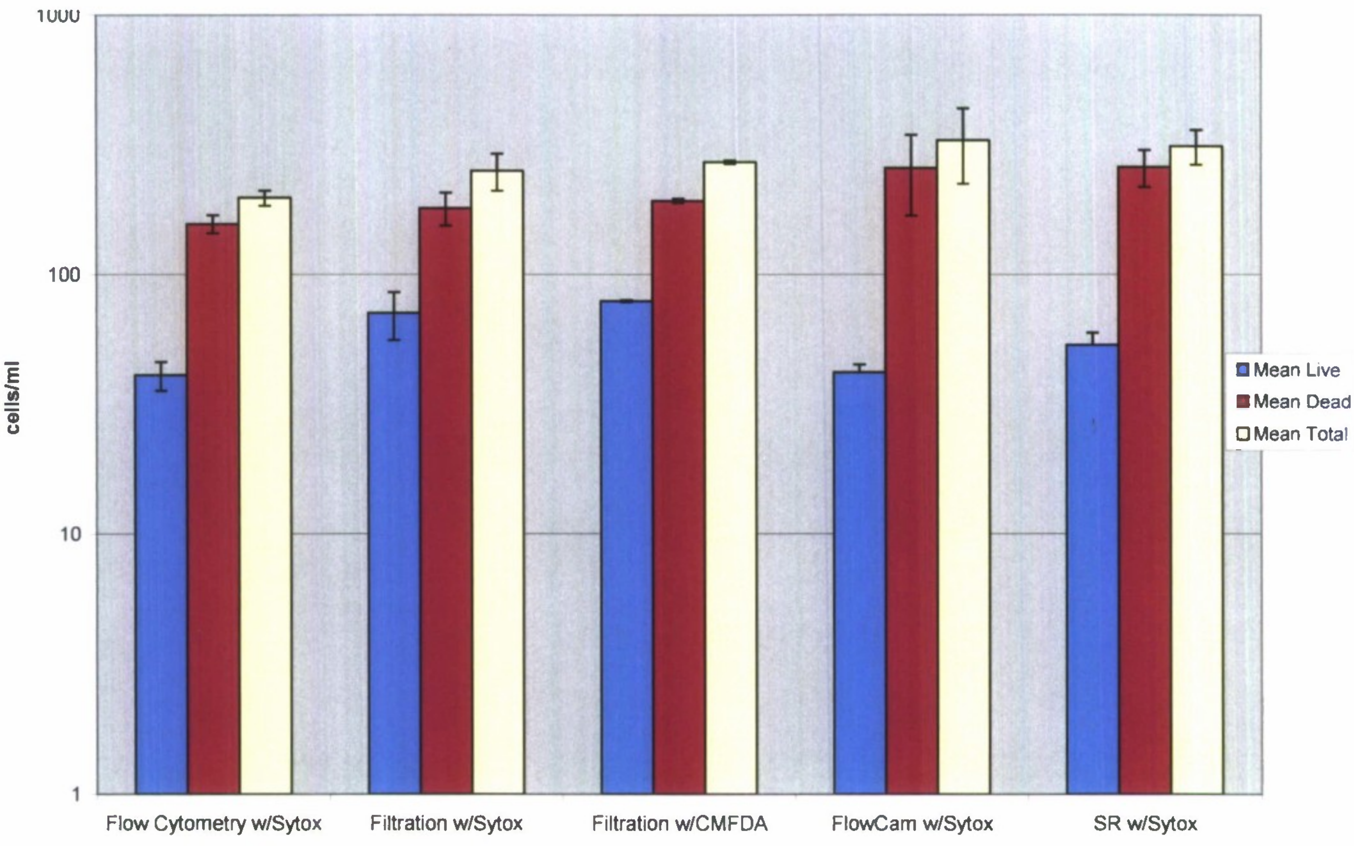

Figure 42. Comparison of different workshop methods for Sample # 2 - the 2 PM January 11, 2008 workshop sample.

3.6.3 Sample # 3 - 10:00 AM Sample from January 12, 2008 – Target Test Sample Concentration 141 Dead: 141 Live Cells

Figure 43 provides a summary of the results reported by each team using the various workshop methods for Sample 3. Again, MLML only utilized SYTOX® Green stain when analyzing this workshop sample using their Flow Cytometer.

The ANOVA analyses indicate significant differences among methods for reported live, dead, and total phytoplankton concentrations from this test. The filtrations results are highest for live plankton and the Sedgwick Rafter (SR) results are highest for dead plankton. For total plankton, the averages are spread out over about a two to one range. That may not be a large spread in practical terms, but it is large relative to the differences among replicates within methods. As indicated in the figure, the live phytoplankton counts were within a factor of three of each other. The total counts are also within a factor of two of each other. The largest variation is in the dead phytoplankton counts with the FlowCAM® reporting results that were significantly lower than the other methods. It is important to note, however, that the FlowCAM® reported "unknown" cells for this workshop sample that were significantly higher compared to all of the other workshop samples.

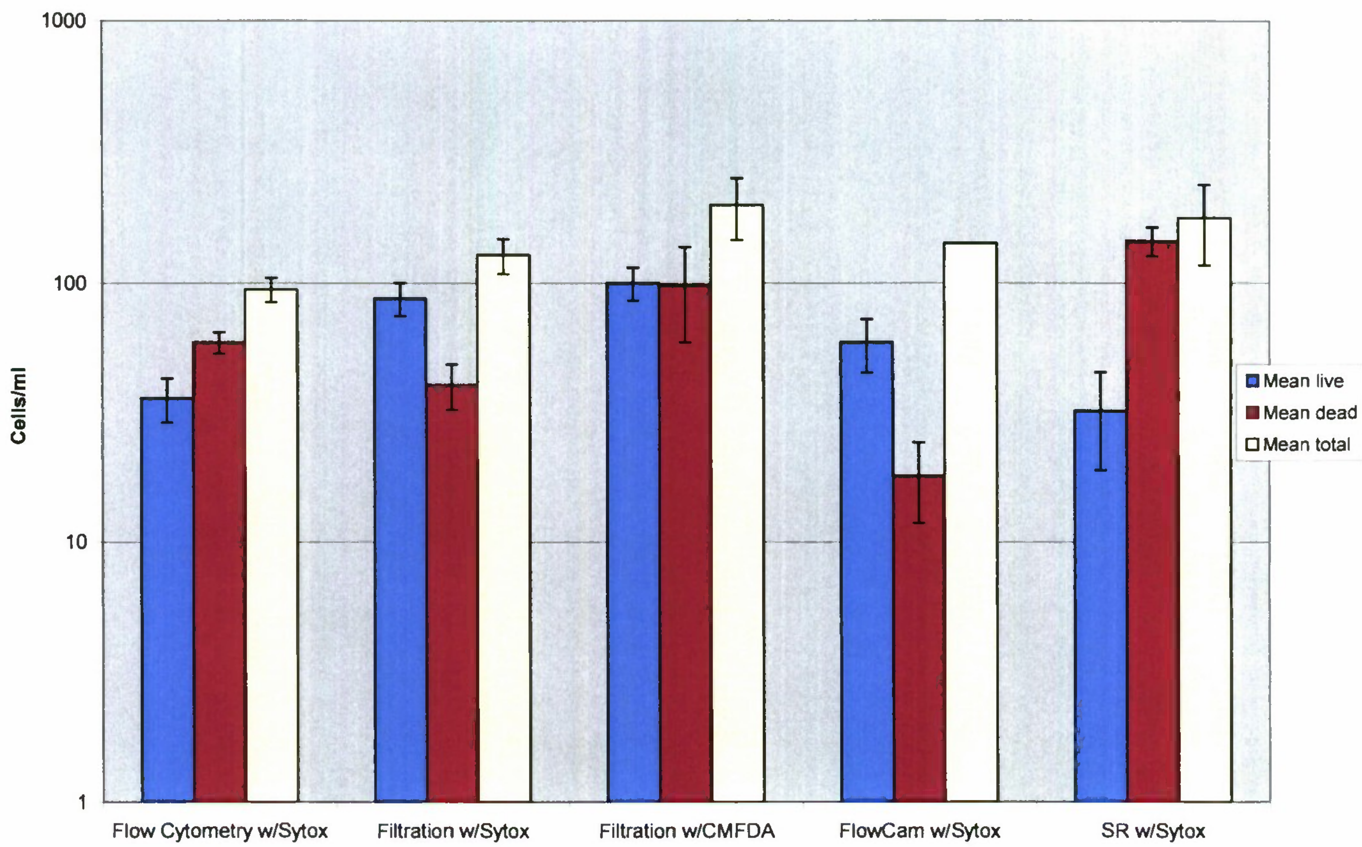

Figure 43. Comparison of different workshop methods for Sample # 3 - the 10 AM January 12, 2008 workshop sample.

3.6.4 Sample # 4 - 2:00 PM Sample from January 12, 2008 – Target Test Sample Concentration 20 Dead: 20 Live Cells

Figure 44 provides a summary of the results for Sample # 4 - the 2:00 PM, January 12 sample. MLML did not analyze this sample using their Flow Cytometer.

There are no significant differences among methods for either live or dead plankton counts. The FlowCAM® results for total phytoplankton concentrations are high. For this test, the number of unknown phytoplankton reported in the FlowCAM® was high (26 +/- 24 mL^{-1}) compared to the number of dead and live phytoplankton results. These cells (which are classified as phytoplankton through manual analysis) account for the difference between the different methods for the total cell concentrations reported for this workshop sample.

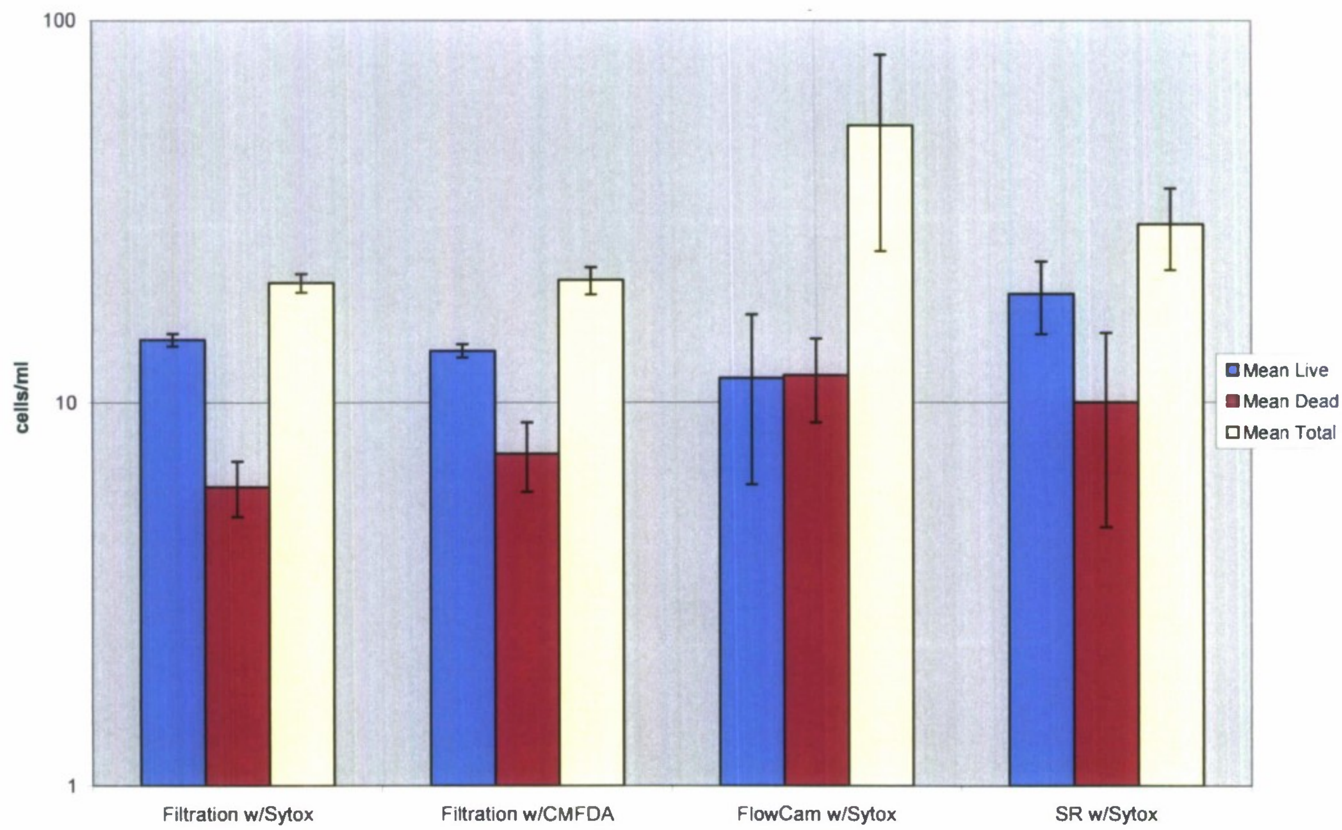

Figure 44. Comparison of different workshop methods for Sample # 4 - the 2 PM January 12, 2008 workshop sample.

3.6.5 Sample # 5 - 10:00 AM Sample from January 13, 2008 – Target Test Sample Concentration 200 Dead: 20 Live Cells

Figure 45 provides a summary of the results reported by each team for Sample # 5 - the January 13 10:00 AM sample. For this test, the different methods counted about the same number of plankton but classified them differently. The ANOVA analyses showed significant differences among methods for both live and dead cell concentrations for this test. As can be observed in the figure, the live cell concentrations were highest for filtration using SYTOX ® Green stain, but the corresponding dead cell concentration is lowest among the methods. There are no significant differences in the total counts. It should be noted that all of the reported results from the different methods are very nearly within a factor of two of each other for the live, dead and total phytoplankton concentrations.

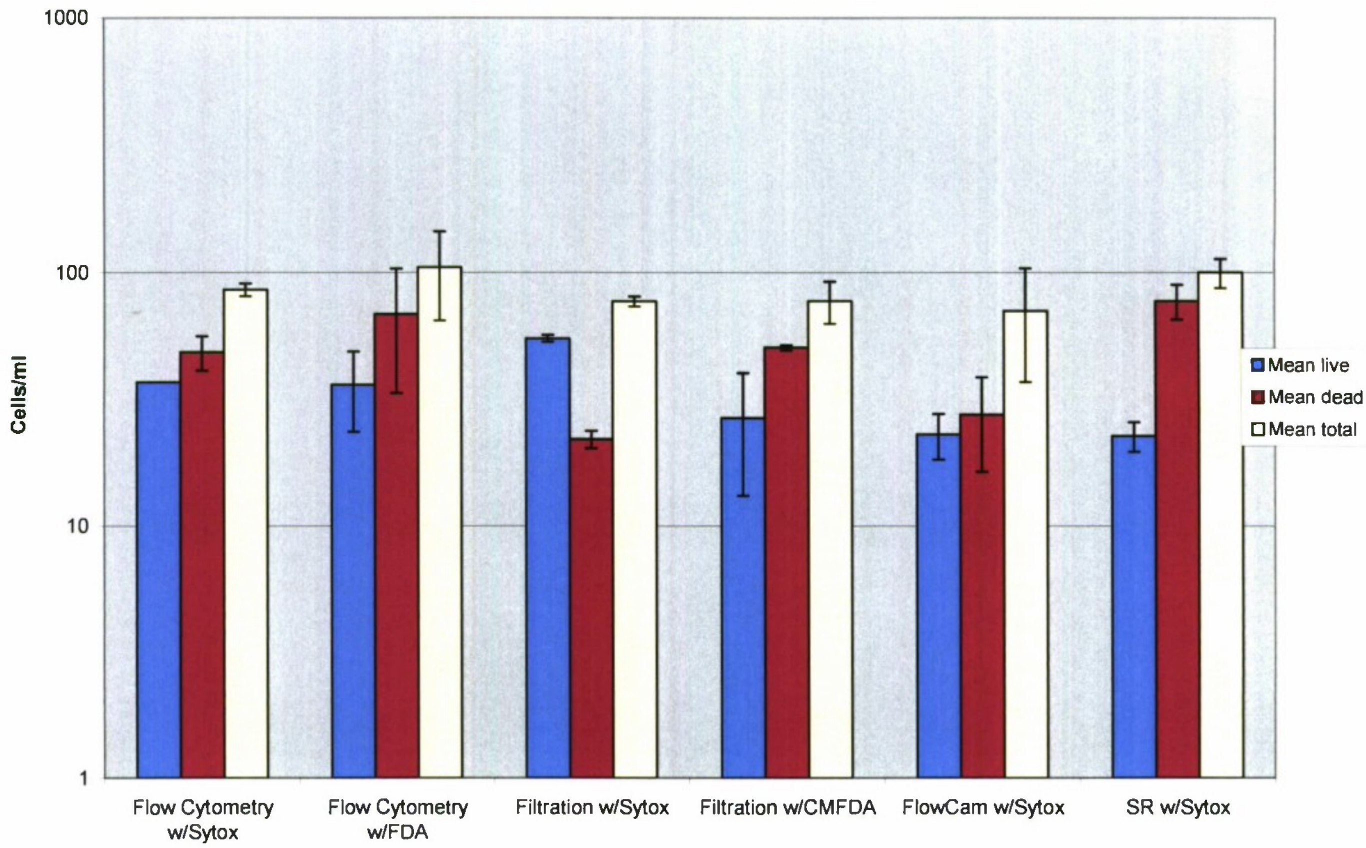

Figure 45. Comparison of different workshop methods for Sample # 5 - the 10 AM January 13, 2008 workshop sample.

3.6.6 Sample # 6 - 2:00 PM Sample from January 13, 2008 – Target Test Sample Concentration 1000 Dead: 20 Live Cells

Figure 46 provides a summary of the results reported by each team for Sample # 6 - the January 13 2:00 PM sample. A review of the data shows that the filtration results are higher than those from the other methods for the live, dead and total phytoplankton concentrations. Within the filtration results, the reported live phytoplankton concentrations were significantly higher for SYTOX ® Green stain than with the Cell Tracker™ Green CMFDA stain. For dead cell concentrations, this trend was reversed. Consequently, there is little difference in the total cell concentrations reported by the filtration methods. Each of the other four methods reported results that were fairly similar to each other. The major difference between the other four methods was that the Sedgwick Rafter reported a significantly lower result for live phytoplankton concentrations and a significantly higher dead phytoplankton concentration. It should be noted that both manual microscopy groups reported problems analyzing this workshop sample.

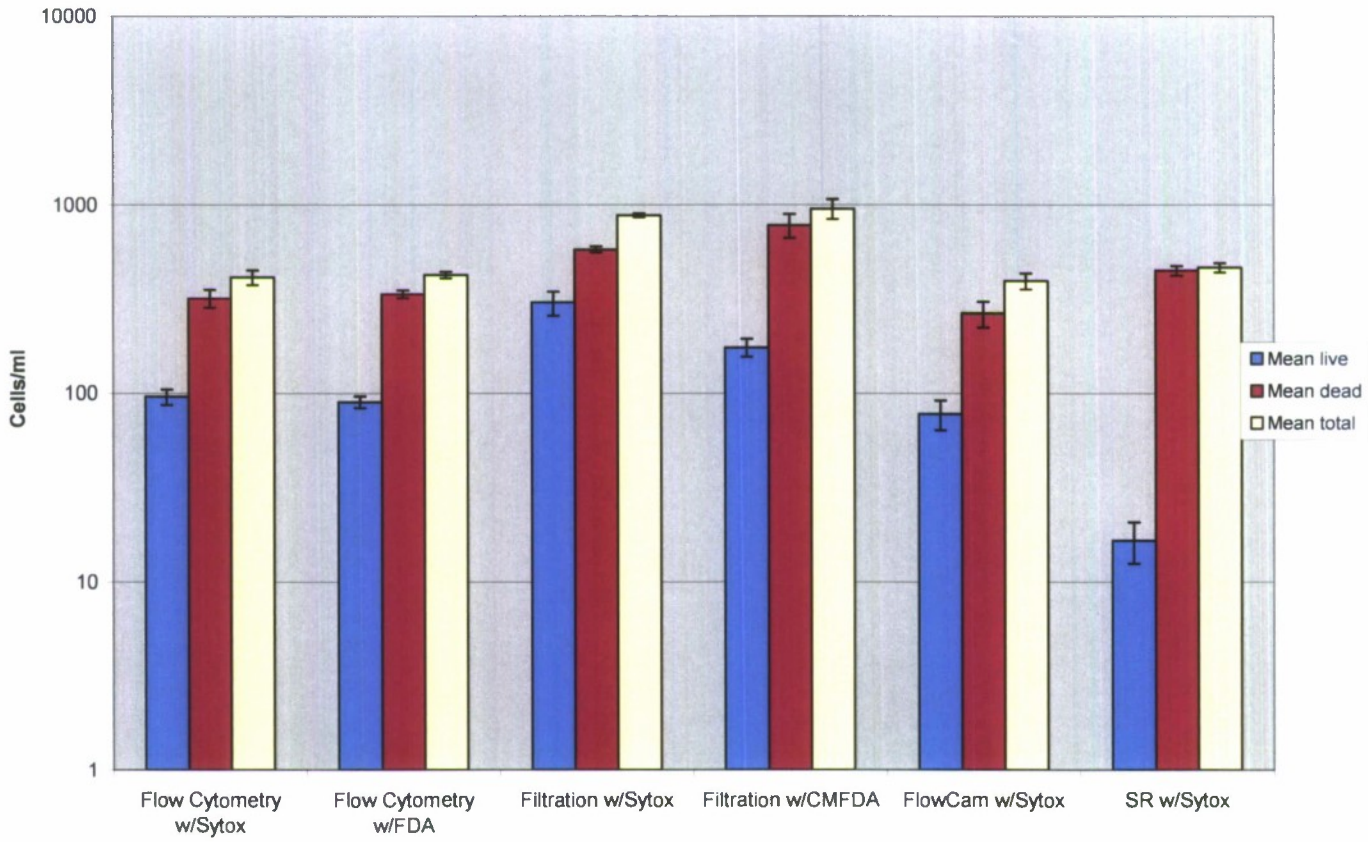

Figure 46. Comparison of different workshop methods for Sample # 6 - the 2 PM January 13, 2008 workshop sample.

3.6.7 Sample # 7 - Sample from January 14, 2008 – Target Test Sample Concentration 20 Live Cells With TSS and TOC Added

Figure 47 provides a summary of the results reported by each team for Sample # 7 - the January 14 complex sample with added POC, TOM and MM. As discussed earlier the FlowCAM® methods that were used for analyzing other workshop samples could not be used with this workshop sample. This was because of the effect of the addition of non-neutrally buoyant particles (test dust) to this workshop sample. Consequently, FlowCAM® only reported a total phytoplankton cell concentration for this workshop sample. The ANOVA analysis showed that the Sedgwick Rafter method ("SR w/Sytox®" in Figure) reported significantly higher concentration for live and total phytoplankton. It also produced the highest result for dead phytoplankton concentrations. However, a similar dead cell result was reported by the filtration using SYTOX® Green stain for this workshop sample.

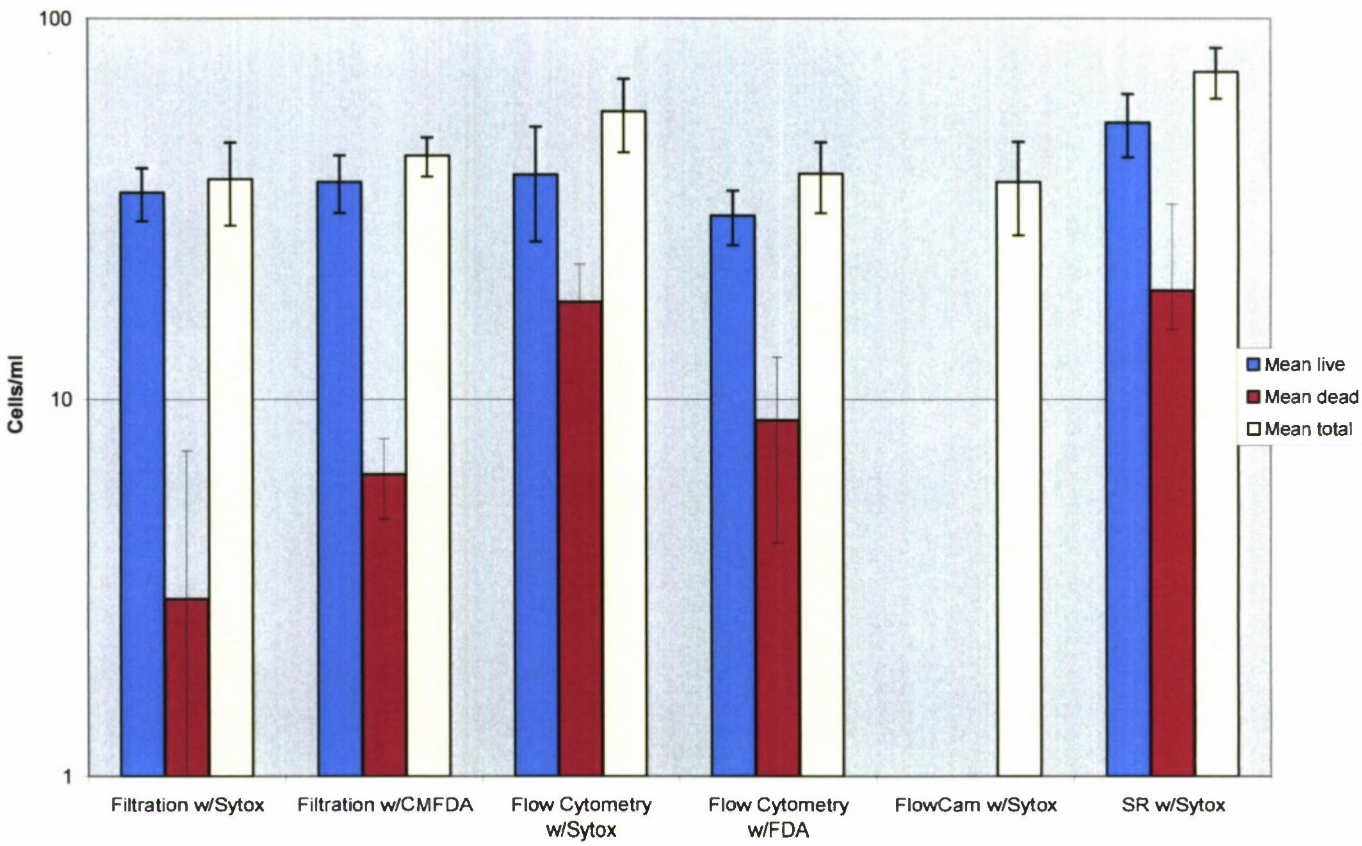

Figure 47. Comparison of different workshop methods for Sample # 7 - the January 14, 2008 workshop sample with added POC, TOM, and MM.

3.6.8 ANOVA Method Precision Evaluations

To evaluate the precision of each measurement method, ANOVA was applied to the data from all seven tests. Since plankton counts vary over nearly three orders of magnitude across tests, the square root transformation was applied to stabilize the variances.

Table 5 summarizes the results of using ANOVA across tests separately for each measurement method. The mean square error (MSE) for each method is the pooled estimate of the residual variance after fitting the means for each test. The MSE results are reported separately for live, dead and total plankton concentrations in the table. DF in the table refers to the degrees of freedom associated with the MSE calculations.

To make use of the table, consider the following. If the replicate results are normally distributed with possibly different means for the different tests, but with common variance σ^2 (precision), then MSE/σ^2 follows a chi-square distribution with the degrees of freedom associated with the MSE calculation. If two methods have the same precision ($\sigma_1^2 = \sigma_2^2$), then $[MSE_1/\sigma_1^2]/[MSE_2/\sigma_2^2] = MSE_1/MSE_2$ follows an F distribution with numerator and denominator degrees of freedom from the associated chi-squares. This value can be looked up in standard F distribution tables. These ratios can be used to test for significant differences between method precisions. Ratios too large or too small to be consistent with the F distribution indicate differences between methods.

Table 5. Residual variability for each method - square root transformation.

Method	Live DF	Live MSE	Dead DF	Dead MSE	Total DF	Total MSE
Flow Cytometry (SYTOX® Green)	23	0.612	23	0.478	23	0.475
Flow Cytometry (FDA)	12	0.426	12	1.58	12	1.41
Filtration (SYTOX® Green)	12	0.536	12	0.591	12	0.484
Filtration (Cell TrackerTM Green CMFDA)	12	0.563	12	1.48	12	1.63
FlowCAM® (SYTOX® Green)	19	0.506	19	1.65	22	1.10
Sedgwick Rafter (SYTOX® Green)	14	0.458	14	1.03	14	0.805

The MSE values for live phytoplankton concentrations in Table 5 are very consistent across methods. The largest difference is between the flow cytometry results with the two different staining methods. The ratio is 0.612/0.426 = 1.44. From statistics tables, the ratio $F_{23,12}(1.44) = 0.74$. Since this is a two-sided test, the p-value is $p = 2(1 - 0.74) = 0.52$. This indicates that there is little evidence of a difference between these two methods which showed the largest variation in MSE value. Consequently, there is little evidence for differences in any of the methods for detecting live phytoplankton cells. This is significant in that evaluation criteria for BWTE is generally specified in live cell concentrations following treatment.

The differences in the MSE values are much larger for dead plankton counts. The precision estimates of the flow cytometry and filtration methods with SYTOX® Green stain for measuring dead cell concentrations are comparable to those for measuring live phytoplankton concentrations. However, the estimates for flow cytometry with FDA, filtration with Cell Tracker™ Green CMFDA, Sedgwick Rafter with SYTOX® Green and FlowCAM® using SYTOX® Green are between two and three times higher for dead plankton as for live plankton. The differences between live count variability and dead count variability, again based on F tests, are statistically significant at the .05 level for flow cytometry with FDA ($p = .03$) and the FlowCAM® ($p = .01$). These results indicate that filtration and flow cytometry using SYTOX® Green stain provided a

more consistent result than the same methods using vital stains. It is also important to note that manual analysis of the data is required to classify dead phytoplankton cells using the FlowCAM®.

For total plankton counts, the estimated variances for the flow cytometry and filtration with SYTOX® Green stain produced results similar to those obtained for both live and dead plankton counts. The FlowCAM® total phytoplankton MSE is between the live and dead values. There is less correlation among the three sets of measurements for FlowCAM® than for the other measurement methods. The other methods classified all plankton as either live or dead. The FlowCAM® results included an unknown category which is included in the total counts but not the live or dead counts.

The apparent differences in precision for dead and total phytoplankton concentrations can still be attributed to a relatively small number of observations, with the variability obtained in particular test using a given method having a significant impact on the reported MSE values.

4 CONCLUSIONS AND RECOMMENDATIONS

Techniques and standardized methods for the enumeration and viability analyses of phytoplankton at low concentrations remain an active avenue of investigation. To date, there are no widely accepted or standardized protocols, as these analyses are complicated by the sparse populations of complex assemblages and the need for precise and accurate measurement of the quantity of living organisms, as opposed to separate analyses of viability (by growth) and quantities (by settling and enumeration). Several of the most promising techniques from NRLKW, WHOI, and MLML were empirically evaluated and compared.

Overall, from the ANOVA work, there is little evidence for differences in any of the methods for detecting live phytoplankton cells. This is significant in that evaluation criteria for BWTE are generally specified in live cell concentrations following treatment. However, the Sedgwick rafter method had the smallest mean percent difference from the expected (target) concentration for live cells. The FlowCAM® showed the next smallest mean percent difference. From the ANOVA for dead cells, filtration and flow cytometry provided more consistent results with SYTOX® Green than they did with vital stains.

A statistical analysis of the results found that while sometimes there were significant differences amongst the techniques, no trend could be established. Therefore, all of these methods are recommended for use, but BWTTFs should be aware of the limitations of these measurement methods and utilize at least two of these techniques for all phytoplankton analyses.

A total of six methods were evaluated in support of the Phytoplankton Enumeration and Evaluation Workshop. A summary of the methods and findings from the workshop follows:

4.1 Conventional Microscopy using Sedgwick Rafter Slides and SYTOX® Green Stain

This method utilizes manual microscopy using an Epi-fluorescence microscope and Sedgwick Rafter slides. A 1-mL volume sub-sample is examined over a 20 row x 50 column grid pattern. Prior to counting, the samples are stained using SYTOX® Green, a fluorescent stain that binds to nucleic acids within a cell. As a cell membrane impermeant stain, the SYTOX® Green only stains cells with compromised membranes. Consequently, the SYTOX® Green fluorescence can be used to identify cells with compromised membranes and which are considered non-viable. By counting the total number of cells as well as those stained with SYTOX® Green, it is possible to determine the live and dead concentrations.

A major advantage of manual microscopy using Sedgwick Rafter slides and SYTOX® Green vital stains is that the instrumentation required to utilize this method are available in many laboratories. Further, sample incubation times are relatively short (30 minutes including settling times) and results can be generated fairly rapidly. Major disadvantages of this method are that it takes several hours (4-5 hours) to characterize a sample (3 sub-samples) and there is no archive of the observations produced. The manual counts are additionally subject to errors based on operator specific biases as well as from fatigue effects during times of extended microscope observations.

The ANOVA analyses performed shows that this method was most precise in measuring the live phytoplankton concentrations in the workshop samples and that results obtained using this method compared favorably to those generated by the other workshop methods.

4.2 Filtration Using SYTOX® Green & Cell Tracker™ Green CMFDA Stains and Epi-Fluorescent Microscopy (Two Methods)

This technique utilizes triplicate 5 – 10-mL sub-samples for each stain used. The samples are stained, incubated, fixed using formalin and then filtered onto a 5-µm 25mm Whatman Cyclopore polycarbonate track-etched membrane filter. The filter is then placed on a plain glass microscope slide and 25 µL of glycerin is added to the center of the filter and the filter covered with a 25-mm square cover glass. Specimens are stored cold and dark until they can be microscopically analyzed.

In support of this workshop all cell counts were made at a total magnification of 200X as it was determined that less magnification would compromise the ability to accurately detect 10µm cells, whereas use of a higher power would increases the amount of time required to enumerate each sample without any appreciable benefit in counting accuracy. Samples can be stored refrigerated for several days before analysis, if necessary; however, it is best to observe them as soon as possible after processing. As was the case with the Segewick Rafter methods, both total and either live (with Cell Tracker™ Green CMFDA) or dead cells (with SYTOX ® Green stain) were separately counted.

There are several key advantages of this particular method in comparison to the other methodologies described in this section. These include the ability to easily process (label) different volumes of samples; flexibility in sample enumeration; unambiguous detection of live and dead cells; chlorophyll signal left intact to aid in the enumeration of dead cells, low cost of materials; simple laboratory protocols; processed sample stability; ability to microscopically inspect the sample population following sample processing, and the possibility of simultaneously dual labeling a sample with live and dead vital stains (if a stain such as Cell Tracker™ Blue CMAC is utilized). Further, multiple rinsing steps of the subsample container and the filtration apparatus assure that the majority of the cells are presented to the membrane filter. This may provide an explanation of why the reported concentrations with this method were higher than those reported by other methods.

Some disadvantages to this method are: labeling variability with live vital stains; moderate daily sample throughput; use of hazardous chemicals; and the cost of vital stains if large sample volumes are processed.

The ANOVA analyses that were performed indicate that filtration with SYTOX® Green stain provided a more consistent measurement than filtration and Cell Tracker™ Green CMFDA stain. Filtration using SYTOX ® Green stain produced similar MSE values for the detection of live, dead and total phytoplankton cells. The ability to effectively work with larger sample volumes and therefore the ability to count a larger

number of cells with this method has the potential to provide significant benefits when working with the low concentration samples that are associated with BWTE evaluations.

4.3 Flow Cytometry using SYTOX ® Green and FDA Stains (Two Methods)

This method utilizes a conventional bench top flow cytometry with either SYTOX® Green or FDA stains. Five replicate subsamples of 1 mL are each analyzed over 15 minutes with a flow rate of 60 µL min^{-1}. Sub-samples are stained with SYTOX® Green Stain or Fluorescein Diacetate (FDA) and incubated prior to their introduction to the flow cytometer.

Following incubation, the stained samples of 1 mL are individually introduced into a flow cytometer which measures green fluorescent intensity (from SYTOX® Green Stain or FDA stain fluorescence) and red fluorescent intensity (from chlorophyll *a* autofluorescence) as a function of particle size. A shift in fluorescent intensity between unstained and stained samples allows for the identification and quantification of live or dead cells, depending on the stain used.

The primary advantage of this technique is the combined ability to determine discrete sizes and cell viability of discrete size categories. In addition, it is not a labor intensive measurement relative to microscopy. Both equipment operation and data analysis require knowledgeable research personnel. Specifically, with respect to the latter, the classification of viable particles from other dead or inorganic particles may be ambiguous and require skilled, technical judgment when the instruments are configured for measuring phytoplankton concentrations.

The ANOVA analyses indicated that flow cytometry with SYTOX® Green stain provided a more consistent measurement than flow cytometry with FDA stain. Flow cytometry using SYTOX® Green stain produced similar MSE values for the detection of live, dead and total phytoplankton cells. The MLML team, on the other hand, reported that it was easier to work with the last more complex sample when the flow cytometer was used with FDA stain. However as mentioned above, the ANOVA analysis indicate more consistent results with SYTOX® Green.

4.4 FlowCAM® Using SYTOX® Green Stain

The FlowCAM® produces a high resolution image of each detected particle as well as two fluorescence intensity signals - one at the chlorophyll *a* auto-florescence wavelength and a second at a wavelength optimized for compatibility with the SYTOX® Green stain (as well as FDA and Cell Tracker™ Green CMFDA stain - and a forward scatter intensity signal. One-milliliter samples are stained, incubated and introduced into the FlowCAM® at a flow rate of approximately 77 µL min^{-1}.

As configured for the workshop FlowCAM® produces a Chlorophyll a fluorescence signal, a SYTOX® Green fluorescence signal, a forward scatter signal as well as significant geometrical information (e.g. Equivalent Spherical Diameter (ESD), minimum and maximum dimensions, etc.) for each detected particle. The particle geometrical data are extracted from the high resolution image of the particle that is captured by the instrument and is believed to be highly accurate compared to estimating this parameter based on forward scatter intensity measurements.

The major advantage of the FlowCAM® is that it collects images of each particle it detects, allowing the operator to verify that detected particles are the result of phytoplankton cells of a particular type. Further,

additional information from the detected particle can be extracted from the images using image processing methodologies, which can result in an improved means for automating the classification of phytoplankton. This capability is not provided in standard flow cytometers. However, as discussed below, the quality of fluorescence intensity and forward scatter intensity measurements in the FlowCAM® is sacrificed to some degree compared to those measured by conventional bench top flow cytometers.

There are several disadvantages of using the FlowCAM® in its current revision to analyze phytoplankton samples. First, the system still does not provide reliable and repeatable measurements of chlorophyll *a* and SYTOX® Green fluorescence intensity levels or forward scatter intensity levels. This inaccuracy makes it difficult to separate phytoplankton cells from other particles in the sample and also makes it much more difficult to automate the detection and classification (both type and viability) of phytoplankton. However, it should be pointed out that even with this limitation, live cell counts could be directly measured by identifying the cells that showed chlorophyll *a* fluorescence but no SYTOX® Green fluorescence. The ANOVA analyses that were performed showed that the FlowCAM® was capable of measuring live phytoplankton concentrations with the same precision as the other workshop methods for six of the seven workshop samples.

The detection of dead cells, however, required more detailed analyses including the identification of phytoplankton through the examination of the particle images. There were also detected phytoplankton whose viability was classified as unknown because these particles did not show a measurable chlorophyll *a* fluorescence or SYTOX® Green fluorescence. The ANOVA analysis showed that these measurements requiring manual interpretation of the data were not made with the same precision as the live phytoplankton concentration methods and that the measurements of dead and total cell concentrations were more variable than those obtained with the other methods.

Non-neutrally buoyant particles are also problematic with the FlowCAM® as a result of its flow cell configuration and its method for introducing samples to this flow cell. This limitation precluded the determination of cell viability in the seventh more complex workshop sample. With its present fluidics configuration, the FlowCAM® can not be recommended for characterizing BWTE samples that have added test dust or other non-neutrally buoyant particles. A flow cell that provides a geometry that allows single particles to be interrogated at a time would help to overcome this difficulty, but it also would likely significantly increase the time required to process a sample, as flow cells of this geometry would have slower sample throughput.

A flow cell that allows the entire flow cell's width to be imaged when using a 20 X objective would also be advantageous for phytoplankton concentration measurements. The 10X objective used in support of this workshop compromises the spatial resolution of the particle images and renders it difficult—using either manual or automated methods—to unambiguously differentiate phytoplankton from test dust and other particles. This type of flow cell would additionally result in slower sample throughput.

In any case, given these deficiencies, when using the FlowCAM® the automated analyses packages should not be relied upon and it is recommended that skilled, technical subject matter experts who understand the devices limitation be responsible for developing protocols and overseeing the instruments operation.

It should again be stated that the FlowCAM® method was scrutinized more strictly than were other methods.

5 REFERENCES

Brussaard CPD, Marie D, Thyrhaug R, Bratbak G (2001) Flow cytometric analysis of phytoplankton viability following viral infection. Aquatic Microbial Ecology, Vol. 26: 157-166.

Lemieux, E.J., Nelson, B.N., Riley, S., Robbins, S.(2007) Development Of Automated Methods For The Enumeration And Classification Of Zooplankton And Phytoplankton Samples, Final Report, U.S. Coast Guard R&D Center, Groton, CT.

Nelson, B.N., Riley, S, Hogan, K, Lemieux, E.J. (2007) FlowCAM® Modifications and Results, BWTTF Position Paper, Naval Research Laboratory, Key West, FL

Veldhuis MJW, Kraay GW, Timmermans KR (2001) Cell death in phytoplankton:correlation between changes in membrane permeability, photosyntheticactivity, pigmentation and growth. European Journal of Phycology, Vol. 36:167-177.

APPENDIX A. WORKSHOP DESCRIPTION PROVIDED TO RESEARCH TEAMS

Phytoplankton Enumeration Experiments Test Plan

Naval Research Laboratory
Key West. FL
November 2007

Background

From January 6-16, 2008, NRL will sponsor Phytoplankton Enumeration Experiments at its facility in Key West, FL. The purpose of these experiments is to provide researchers with test samples of known phytoplankton concentrations to allow them to demonstrate methods for accurately enumerating viable phytoplankton concentrations over a wide range of concentration levels in increasing complex samples. The overall objective of these tests is not to show that the method can identify which sample is which, but to demonstrate the complete enumeration and classification process including the number of measurements required and how these measurements are combined to develop a single viable cell concentration for each sample analyzed.

Testing at the NRL Ballast Water Treatment Test Facility (BWTTF) necessitates the complete characterization of a significant number of samples to determine the efficacy of ballast water treatment equipment (BWTE). To document biological treatment performance, concentrations and viability of phytoplankton must be determined before and after treatment. During such assessments of BWTE efficacy, significant man-hours are expended determining phytoplankton concentrations and viability in challenge and post-treatment samples.

The characterization of these samples is compounded by other requirements for the challenge waters used in support of standardized tests. Additional water parameters that can make sample characterization more difficult include total dissolved solids and suspended sediments in the same size range as phytoplankton in the challenge and post-treatment samples.

The current methods used for enumerating viable phytoplankton in challenge and post-treatment samples at the BWTTF involve the analysis of nine separate 1-mL phytoplankton samples that are taken from one 3-m^3 sample tank. These characterizations are performed using an epi-florescent microscope and samples that are dyed using SYTOX® green (to identify the non-viable phytoplankton). It currently takes approximately 4½ hours of continuous observations to enumerate, classify and determine the viability these nine phytoplankton samples using these methods.

The testing performed at the BWTTF has identified a need for methods that allow the more time efficient, but still very accurate characterization of phytoplankton samples. Specifically, this work has identified the need for robust and time efficient measurement methods for characterization of samples in which there are 10 or fewer viable organisms present mL^{-1}.

The experiments that will be conducted are directed at providing researchers with samples to allow them to demonstrate enumeration methods that identify viable phytoplankton in the 5 – 15 micron size range over a range of concentrations that are relevant to standardized testing and with varying complexity.

It is desired to maintain a workshop atmosphere during these ten days. We believe that we have allowed sufficient time for group discussions on the various measurement techniques. We are especially interested in engaging in discussions of the methods that each group uses for sub sampling the larger samples and for generating a single concentration level from the individual measurements.

Phase I Experimental Outline

The purpose of the Phase I experiments is to provide researchers with relatively homogenous samples to demonstrate their enumeration methodologies. Although two surrogates are used in support of standardized testing, it has been decided to perform the Phase I experiments with phytoplankton samples that are generated using a single phytoplankton species (*Tetraselmis*). Table 1 below provides the test matrix for Phase I Experiments.

Table A-1. Seven samples types used in support of the Phase I Experiments.

Concentrations	Dead:Live Ratio	Dead:Live Ratio	Dead:Live Ratio
1 /ml	0:1	10:1	100:1
10/ml	0:1	10:1	100:1
100/ml	0:1	X	X

The Phase I Samples will cover three viable cell concentration ranges and three dead:live ratios (for two of the concentration ranges).

Seven total samples will be used in support of the Phase I experiments to assess the various measurement methods. The samples will be prepared using artificial seawater and with pure phytoplankton cultures. More details of the procedures that will be used for developing these samples will be provided in a separate document in approximately three weeks.

Each of the researchers participating in this experiment will receive samples that are prepared by NRL Key West consistent with the concentrations and dead:live ratios that are outlined in the Table 1. For each of these concentrations and dead:live ratios, 1 L of sample will be provided to the researchers without their knowledge of the sample concentration or dead:live ratios. The researchers will determine how to optimally subsample the 1 L sample and use their enumeration methodology to determine the viable cell concentrations in each of the samples provided. It is expected that the researchers will provide details on how the sample was subsampled, how the subsamples were analyzed and how the subsample measurements were combined to generate a single concentration value for each sample analyzed. If appropriate information is collected during the measurements, than statistics that provide an indication of both the accuracy and uncertainty of the measurements should be generated and provided by the researchers.

It is expected that the researchers will be able to characterize at least one sample/day. The Phase I experiments will be conducted over a 7 day period with each group of researchers characterizing at least 1 sample of each type described in Table 1.

Each research team will be responsible for tabulating their results for each of their sample characterizations and for performing appropriate statistical analysis on their data. Researchers should report a concentration for live cells and a concentration level for dead cells. NRL observers will also note the time that is required using each of the methods for both individual measurements (e.g. analyzing 1 mL of the 1 L sample) as well as for the full complete characterization of each of the provided samples.

Phase II Experimental Outline

The purpose of the Phase II experiments will be to provide the researchers with more complex samples for analysis. These samples will be more indicative of the samples that are evaluated during BWTTF standardized testing.

The Phase II experiments will be performed only if time allows during the execution of the Phase I experiments. If a researcher completes the required Phase I analysis with sufficient time remaining then a more complex Phase II sample of the same concentration and dead:live ratio will be provided to the researcher for analysis.

Sediments and dissolved solids will be added at levels that are consistent with those used during standardized testing (4 – 6 mg/L dissolved organic carbon, particulate carbon 4 – 6 mg/L, and mineral matter 8-11 mg/L).

Samples will be prepared according to the concentration levels and dead:live ratios that were previously provided in Table 1. The samples will be prepared using artificial seawater and with pure phytoplankton cultures. Sediment and total dissolved solids will be added to the samples. More details of the procedures that will be used for developing these samples will be provided in a separate document in approximately three weeks.

If time allows, a researcher will receive a Phase II sample that is prepared by NRL Key West that is consistent with the previously analyzed Phase I sample. For each of these concentrations and dead:live ratios, 1 L of sample will be provided. The researchers will determine how to optimally subsample the 1 L sample and use their enumeration methodology to determine the viable cell concentrations in each of the samples provided. It is expected that the researchers will provide details on how the sample was subsampled, how the subsamples were analyzed and how the subsample measurements were combined to generate a single concentration value for each sample analyzed. If appropriate information is collected during the measurements, than statistics that provide an indication of both the accuracy and uncertainty of the measurements should be generated and provided by the researchers.

Each research team will be responsible for tabulating their results for each of their sample characterizations and for performing appropriate statistical analysis on their data. Researchers should report a concentration for live cells and a concentration level for dead cells. NRL observers will also note the time that is required using each of the methods for both individual measurements (e.g. analyzing 1 mL of the 1 L sample) as well as for the full complete characterization of each of the provided samples.

Schedule

January 6 - 8 Arrival and initial work to calibrate and get measurement methods ready. We should be prepared to provide appropriate calibration samples for the researchers during these initial days.

January 9 - 15 Experiments/Discussions.

January 16 – Wrap Up – Discussions- Etc.

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APPENDIX B. WORKSHOP METHODS

B.1 Phytoplankton Sample Preparation

Preparation of test samples at desired live and dead cell concentrations involved the serial dilution of live and dead *Tetraselmis* stocks (strain PLY 429) purchased by NRLKW from Reed Mariculture (Campbell, CA). To reduce the amount of microscope time required by NRLKW to prepare phytoplankton samples, it was decided to use Reed Mariculture's measurements of sample concentration provided with each purchased stock solution of live and dead *Tetraselmis*.

Prior to the start of the workshop, NRLKW demonstrated that using serial dilutions and the concentration values provided by Reed Mariculture allowed preparation of samples with total live or dead cell populations in the 10 cells mL^{-1} concentration range (within a factor of 2). This result demonstrated the concentrations provided by Reed Mariculture were adequate for determining the dilutions required to produce the test samples of approximately known values to the workshop participants. The major advantage of this approach is that it required no additional microscope time. This in turn allowed NRLKW personnel to participate in the workshop by analyzing test samples using the methodologies previously employed during BWTE evaluations at NRLKW.

Initial work performed prior to the start of the workshop also showed the dead cells, which were killed by centrifugation, could be successfully stained using the DNA stain SYTOX® Green. At the start of the workshop, additional microscope observations of the phytoplankton cells from the dead stock using an epifluorescence microscope showed that the dead cells were autofluorescing in the green region of the spectrum. This autofluorescence was observed on the outside of the cells. The signal from this fluorescence would initially mask the cell's chlorophyll *a* autofluorescence signal and would directly interfere with the SYTOX® Green fluorescence. Interestingly, as the cells were observed over a period of two minutes, it appeared the illumination from the epifluorescent microscope broke down the compound on the outside of the cell that was autofluorescing. When breakdown occurred, the cells' chlorophyll *a* autofluorescence could be readily observed. It should be noted that only cells that were in the microscope's active field of view went through this transition.

Because the dead cells autofluoresced, a new method for preparing dead phytoplankton stock for the workshop was developed using ultraviolet irradiation. Dead, UV-treated cells did not autofluoresce and could be stained with SYTOX® Green to indicate cell viability. Unfortunately, an unanticipated outcome is that it is likely that variable rates of cell survival from the irradiation process impacted NRLKW's ability to prepare samples with accurate numbers of live and dead cells.

B.1.1 Sample Preparation Procedures

Basic Sample Preparation

The first step in generating the workshop samples involved making artificial seawater. To make each batch, a 20-liter (L) plastic, freshly washed Nalgene® container was used. To this container, 10 L of Type II distilled water and 2.5 cups of Instant Ocean® were added to attain a salinity of 30-31 practical salinity units (psu)). The water within the Nalgene® container was then mixed until the Instant Ocean® dissolved.

To produce a batch of dead *Tetraselmis* from the live *Tetraselmis* stock received from Reed Mariculture, a UV light (a 48 inch, 30 Watt Ultra Violet Germicidal Lamp) in a bio-hood (Forma Scientific, Inc.) was

turned on and allowed to warm up for 20 minutes. Live *Tetraselmis* stock was removed from an incubator and allowed to warm to room temperature (approximately 30 minutes). The live *Tetraselmis* stock was then mixed within its container by gently inverting the container a minimum of 10 times.

After mixing, a pipette was used to transfer three, 20-milliliter (mL) aliquots of live, concentrated *Tetraselmis* stock to individual plastic Petri dishes. Next, the three Petri dishes were placed within approximately 13 cm of the UV light and remained under the UV lamp, uncovered, for 30 minutes. After UV exposure, the Petri dishes were removed from the lamp and allowed to sit on the lab bench for 60 minutes. Based on other workshop participants' experience in using UV to kill phytoplankton, it is believed that this provided sufficient time for the majority of the phytoplankton to die following the UV irradiation.

The dead *Tetraselmis* stock was removed from the Petri dishes using a pipette and transferred to two 50-mL plastic containers. This concentrated dead stock was then used to create the workshop test samples. A calibrated 10-L graduated cylinder was used to accurately measure 10 liters of artificial seawater and to transfer it to a 20-L Nalgene® container. The stock concentration values from Reed Mariculture were then used to determine the volume of live and dead stock solution to be added to the 10 L volume to obtain the desired cell concentration levels and dead:live ratios. Prior to adding the stock solutions to the 10 L of artificial seawater, a volume of artificial seawater, equal to the volume of the *Tetraselmis* stock to be added, was removed from the 10 L sample. The algal stock solution was added to the artificial seawater, and the 10 L sample was mixed through a series of inversions.

The final step of the sample preparation process involved measuring the volumes from the 10-L sample for each workshop participant. Typically, 1 L of test sample was provided to both the NRLKW and WHOI microscopy teams, 300 mL of test sample was provided to the NRLKW/Fluid Imaging Technologies team, and remaining sample volume (~ 7.5 L) was provided to the MLML team.

Total Suspended Solids/Total Organic Carbon (TSS/TOC) Preparation Method

The final phytoplankton sample provided to the teams was more complex than the previous samples. To make this sample representative of samples processed during BWTE tests at NRLKW, it was prepared with mineral matter, dissolved organic carbon, and particulate organic matter, in addition to a low concentration of live *Tetraselmis* cells. The goal was to achieve a sample with the following characteristics:

- 20 live *Tetraselmis* cells mL^{-1}
- 5 mg L^{-1} Dissolved Organic Carbon (DOC)
- 5 mg L^{-1} Particulate Organic Matter (POM)
- 19 mg L^{-1} mineral matter (MM)

To obtain these water-quality attributes, NRLKW has developed procedures to add decaffeinated iced tea, humic matter, and medium and coarse ISO test dust (Powder Technology Inc., Burnsville, MN) to the artificial seawater. Based on a series of calculations, the following volumes of these materials should have been added to the artificial seawater:

- 47.9 mg Decaffeinated Tea (a proxy for DOC)
- 16.63 mg humic matter (a proxy for POM)
- 10.5 mg Medium ISO Arizona test dust (a proxy for MM)
- 10.5 mg Fine ISO Arizona test dust (a proxy for MM)

However, an error occurred, so the tea, humic material and test dust concentrations added to the artificial seawater were, in fact, 10 times higher than those mentioned above. In order to prevent the clogging of the flow cells used in the FlowCAM® and MLML flow cytometer, it was decided to filter the sample using a 25 µm mesh screen prior to adding the live phytoplankton. The filtering process removed particles larger than 33 µm from the modified test sample. Two liters were filtered using this process. Next, live *Tetraselmis* stock was added to this solution with the goal of creating a test sample with a live cell concentration of approximately 20 cells mL^{-1}. The sample was mixed and as only 2 L of this sample was generated, a smaller but still appropriate sample volume was provided to each of the workshop participants. It is important to note, that the actual sample volume that was analyzed by each of the workshop participants was the same or larger than that analyzed with the other workshop samples. Some additional sample was retained and provided to an outside lab which performed analysis of the TOC, POM and MM concentrations on this workshop sample.

B.2 NRLKW - Microscopy

The Sedgwick Rafter slide is a specialized gridded (20 row x 50 column grid) microscopy slide for organism enumeration of a 1-mL volume sample. Prior to counting, the samples were stained using SYTOX® Green, a fluorescent vital stain that binds to nucleic acids within a cell. As an impermeant stain, SYTOX® Green only stains cells with compromised membranes. Consequently, the SYTOX® Green fluorescence can be used to identify cells with compromised membranes and therefore which are considered non-viable. By counting the total number of cells as well as those stained with SYTOX® Green, it is possible to determine, via subtraction, the live and dead concentrations.

A 1-L workshop sample was provided to the NRLKW Microscopy group. The 1-L sample was first well mixed through a series of sample inversions. A 10-mL subsample was removed and dispensed into an 80 mL beaker. Using a sterile dropper, two drops of low-acidity vinegar (acetic acid) were added to the 10 mL sample to de-mobilize the living *Tetraselmis* cells. The phytoplankton and vinegar were homogenized by pouring the solution back and forth between the beaker and a second 80 mL beaker. After the Tetraselmis sample and vinegar were well mixed, a 1 mL subsample of the de-mobilized Tetraselmis sample was drawn from the 80 mL beaker using an Eppendorf micropipette into a 1 mL centrifuge tube with 10 µL of 0.09 millimolar (mM) concentration SYTOX® Green. The centrifuge tube was mixed using a vortexer, and the tube plus phytoplankton sample and stain were placed in the dark to incubate for 10 minutes (min).

Following incubation, the prepared sample was again mixed using the vortexer, and the 1-mL sample was drawn from the centrifuge tube using an Eppendorf micropipette and dispensed onto the Sedgwick Rafter slide. The cover glass was carefully placed onto the counting slide perpendicular to the long axis of the slide. The cover glass was swung across the counting cell so it completely covered the sample. Careful alignment of the cover glass prevents air bubbles from being introduced into the sample and ensures that the slide contains a true 1-mL sample (Figure B-1).

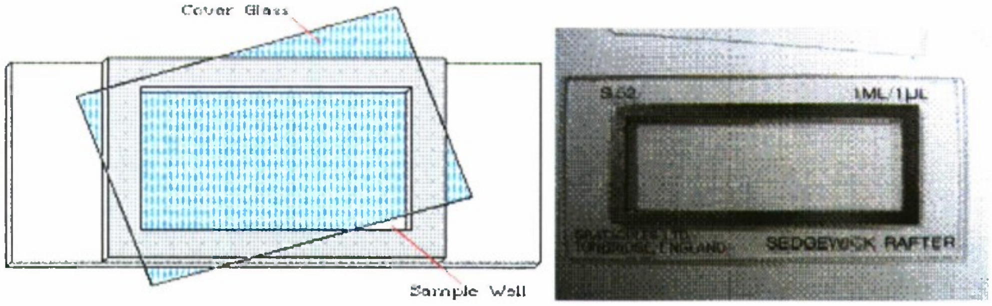

Figure B-1. Sedgwick Rafter counting slide.

Once the sample was prepared on the Sedgwick Rafter slide, it was placed in the dark for an additional 20 min to allow phytoplankton cells to settle on the slide. When the stained *Tetraselmis* cells had settled, the Sedgwick Rafter slide was secured on the base of a Nikon E600 microscope with phase contrast and epifluorescence capabilities. The microscope was then used to identify and count both live and dead (as indicated by SYTOX® Green fluorescence) cells. A data tally sheet that was used to record sample analysis results is provided in Table 2. This sheet was specifically developed for supporting BWTE at NRLKW. Consequently, parameters such as "Sample Port", "Ambient Live" and "Ambient Dead" are not relevant to the analysis of workshop samples. The letters in the Table are randomly selected and correspond to specific rows on the Sedgwick Rafter slide. For each identified letter on the data sheet, the number of live and dead cells were counted and entered into the appropriate column. Target sample live and dead cell concentration levels were entered into the "Sample Port" row during the workshop.

To determine live and dead populations, the *Tetraselmis* cells were first identified and counted on a given row of the slide (total cell count for the row being analyzed). Once this count was completed, the microscope was switched to operate in the epifluorescence mode. This configuration allowed cells with SYTOX® Green staining of their nuclei (e.g., the dead cells) to be quickly identified and counted. The number of live cells in the row was then determined by subtracting the number of dead cells from the total number of cells in the row. This process was repeated for each of the 20 rows on the Sedgwick Rafter slide such that total live and dead cell counts mL^{-1} could be determined. For the majority of the workshop samples, cells in the entire 1-mL Sedgwick Rafter slide volume were counted.

Table B-1. Sample data tally sheet used to record phytoplankton sample data. Note that this sheet was developed to support BWTE tests at NRLKW. As the "Sample Port" designation is not relevant to the analysis of workshop samples, the target sample live and dead cell concentrations were entered into this row. The columns marked Ambient Live" & "Ambient Dead" were not used in this workshop.

Phytoplankton Stained Live/Dead				
Date:				
Sample Port:				
Phytoplankton:				
Run:			Ambient	
	Live	Dead	Live	Dead
D				
B				
Q				
S				
K				
R				
H				
C				
L				
M				

B.2.1 Advantages & Disadvantages to Sedgwick Rafter Methodology

The advantage of manual microscopy using Sedgwick Rafter slides and SYTOX® Green vital stains is that the instrumentation required for this method is available in many laboratories. Further, sample incubation times are relatively short (30 minutes including settling times), and results can be generated fairly rapidly.

The major disadvantages of this method are that it takes several hours (4-5 hours) to characterize a sample (3 subsamples), and there is no archive of the observations produced. Additionally, the manual counts are subject to errors based on operator specific biases as well as from fatigue effects during times of extended microscope observations.

B.3 WHOI – Microscopy

B.3.1 Methods

Workshop samples ranging in volume from 300 mL to 1 liter (L) were provided to the WHOI Microscopy group. When the sample was provided, it was first well mixed by a 360° inversion of the sample container ten times before removing an initial subsample. The sample container was mixed two additional times between each subsample, and 3 subsamples were prepared and counted for each vital stain used except as noted. The subsample volume was usually 5 mL, with a 10 mL subsample used when a lower target cell concentration was anticipated (10 AM sample on January 12). Each subsample was pipetted into a 15 mL disposable centrifuge tube, and an appropriate amount of vital stain added to achieve a final concentration of 5 micromolar (µM) for Cell Tracker™ Green CMFDA (5-chloromethylfluorescein diacetate) and Cell Tracker™ Blue CMAC (7-amino-4-chloromethylcoumarin), or 0.5 µM for SYTOX® Green. (Note: All

vital stain stocks should be resuspended in anhydrous dimethylsulfoxide (DMSO) aliquoted into centrifuge tubes or other containers, and stored frozen at -20°C for long-term storage.) The vital stain and sample were mixed twice by a 360° inversion of the centrifuge tube, and the samples were allowed to incubate in the dark for 45 minutes at room temperature. (Note: The incubation temperature should be the same as the sample maintenance temperature.) Following the incubation, 100% formalin was added to each sample such that a final concentration of 5% v/v was achieved in each sub-sample prepared for analysis. Sub-sample incubation initiation times were staggered to ensure that the sub-samples were not exposed to formalin for more than one minute prior to filtration and that all sub-samples were prepared identically. Longer exposure times to formalin might enhance dead cell numbers if SYTOX® Green is used.

The samples were again mixed twice by 360° inversion, and the contents of the centrifuge tube were filtered onto a 5μm, 25mm Whatman Cyclopore polycarbonate track-etched membrane filter (Whatman Cyclopore catalogue #: 7062-2513) using minimal vacuum pressure. The sample container was rinsed 3 times with filtered seawater (FSW), and the wash water was also added to the filter funnel. The sample was filtered until approximately 1 mL remained in the filter tower, after which the sample was rinsed with approximately 5 mL of FSW 4 times. Following the final rinse, the tower was drained completely and the filter placed on a plain glass microscope slide. Twenty-five μL of glycerin was added to the center of the filter using an air displacement pipette with a wide tip and the filter covered with a 25 mm square, #1 cover glass.

The specimens were stored cold and dark until they could be microscopically examined using a Lietz Diaplan microscope equipped with a 100-watt high pressure mercury light and a Chroma 41012 (excitation: 480/40x, dichroic: 505LP, emission: HQ510LP) filter set for SYTOX® Green and Cell Tracker™ Green CMFDA. For Cell Tracker™ Blue CMAC, a Chroma 11000v3 (excitation: D350/50x, dichroic: 400DCLP, emission: E420LPv2), or the equivalent is recommended for use. All cell counts were done at a total magnification of 200X as it was determined that less magnification would compromise the ability to accurately detect *Tetraselmis* cells, whereas use of a higher power would increase the amount of time required to enumerate each sample without any appreciable benefit in counting accuracy.

The processed sample filters were enumerated several different ways depending upon the total number of target cells seen on the filter. For low-density samples, either the entire filter area or half of the filter area was scanned and enumerated. For high-density samples, 2 or more sweeps across the filter area in a "+" pattern were made until a minimum of 400 individuals were tallied. All cells seen within the field of view (a sweep across the filter) were tallied. The area of a 25 mm diameter filter is 491 mm^2. The "sweep" area at 200X for a 25 mm diameter filter was determined to be 17.5 mm^2 using a stage micrometer. In all situations, only cells on the filter were counted; cells under the coverglass but beyond the bounds of the filter (a rare occurrence) were not considered. Samples can be stored refrigerated for several days before analysis, if necessary, however, it is best to observe them as soon as possible after processing.

B.3.2 Method Advantages/Disadvantages

This method affords the user several key advantages in comparison to the other methodologies utilized during this workshop. These include the ability to easily process (label) different volumes of samples; flexibility in sample enumeration; unambiguous detection of live and dead cells; chlorophyll *a* signal left intact to aid in the enumeration of dead cells; low cost of materials; simple laboratory protocols; processed sample stability; ability to microscopically inspect the sample population following sample processing; and the possibility of simultaneously dual labeling a sample with live and dead vital stains.

Some disadvantages to this method are: labeling variability with live vital stains; moderate daily sample throughput; use of hazardous chemicals and the cost of vital stains if large sample volumes are processed.

As mentioned above, various sample amounts ranging from µL to L volumes can be labeled with live and dead cell vital stains so long as the proper final concentration of stain can be achieved (5µM Cell Tracker™ Green CMFDA and Cell Tracker™ Blue CMAC, or 0.5 µM SYTOX® Green). The volume of sample that should be processed is primarily driven by the expected cell densities and the required measurement accuracy (which increases with the number of cells counted). Other factors, however, such as sample biomass and detrital matter concentrations are also important in determining the specific sample volume to process. Given these factors, a quick microscopic evaluation of an unfiltered sample prior to processing can be used to help define the volume of a particular sample to process.

Once processed, the sample filter can be counted in its entirety or counted partially depending on the selected target cell densities. Andersen and Throndsen (2003) provide a good summary of how to count slides with various sample densities and provide a statistical table documenting the percent error associated with different cell counts. A high-quality microscope is required to quickly, comfortably, and reliably obtain an accurate cell count. Key microscope features to consider are a 100-watt high-pressure mercury light source, tailored optics for fluorescence microscopy, and fine-tuned fluorescence filter sets with long-pass emission filters that allow uninhibited visualization of the live and dead cell fluorochromes in addition to fluorescently active cell pigments. A good-quality, color, digital camera to document experimental results is also suggested.

Long-pass fluorescence emission filter sets are superior to band-pass emission filters in several aspects: they often allow a brighter level of signal to be visualized, they provide a more realistic color of the vital stain fluorescent product, and they allow for all cellular fluorescent colors (e.g. Cell Tracker™ Blue CMAC (blue fluoresence), SYTOX® Green (green fluoresence) and chlorophyll *a* autofluorescence (red fluoresence)) above their cutoff point to be seen simultaneously. The ability to observe these fluorescent signals simultaneously in phytoplankton cells is extremely beneficial when enumerating and classifying the viability samples. Pigment fluorescence in algal cells is usually red (chlorophyll *a*), or orange (phycoerytherin), and this fluorescence can be masked to various levels by live vital stains such as Cell Tracker™ Green CMFDA and Cell Tracker™ Blue CMAC. The pigment fluorescence signals are typically sufficiently strong and easily observed in freshly killed cells that are labeled with SYTOX® Green due to the small size of the nucleus (where SYTOX® Green fluorescence occurs) compared to the cytoplasmic contents of most phytoplankton cells (where pigment fluorescence occurs). In this case, the pigment fluorescence allows for quick recognition of phytoplankton cells with the highly localized and intense SYTOX® Green fluorescence allowing for a rapid evaluation cell viability. Pigment fluorescence can also be used to identify dead cells in a sample labeled with only a live cell vital stain if a companion dead cell stain was not utilized. In this situation, the live cell would be illuminated with the vital stain in addition to some residual pigment fluorescence (if not fully masked), whereas the dead cell would only have pigment fluorescence.

The microscope is a major expense, and thus, a disadvantage associated with this method. Most of the other required equipment needs are low in cost and are usually found in most biological laboratories.

The sample protocols as outlined in the methods sections are simple to follow and do not require a high level of laboratory expertise. Unfortunately, sample throughput is low with this protocol. Comfortably, 6 samples can be labeled and enumerated in 6 hours, but this quantity is dependent on the number of target organisms being enumerated, the complexity of the sample in terms of biodiversity, and sample matrix

factors. A breakdown of the time requirements for this method are as follows: Two hours are required to label 6 samples whether they be 2 separate samples with 3 subsamples each, or 1 sample being labeled with 2 vital stains with three subsamples each, or some other combination ultimately resulting in 6 processed samples. The labeling consists of preparation of the reagents, a 45-minute sample incubation time, preservation and filtration of the samples, mounting the filters on slides and cleanup. The remaining 4 hours is for sample enumeration, including allowances for data entry.

Labeled samples are stable for at least 3 days, and perhaps longer, provided that the samples are kept at 4°C and dark. This provides the potential for analyzing more sub-samples as measurements can be conducted over several days versus several hours. Additional studies should be conducted if long-term sample stability is deemed to be a desired criterion.

It is believed by the authors that samples can be simultaneously dually labeled with live and dead cell stains as long as the reporter fluorochromes are spectrally separated from one another, e.g., using Cell Tracker™ Blue CMAC and SYTOX® Green. This experiment was attempted using SYTOX® Green and Cell Tracker™ Green CMFDA, but the reporter fluorochromes in the two vital stains are too similar in color, and it became difficult to distinguish which vital stain was reacting within a specific cell in many instances because of the variable labeling of Cell Tracker™ Green CMFDA in live cells. This variability is believed to be due to the physiological status of the cell. A cell that is very healthy will exhibit robust Cell Tracker™ Green CMFDA labeling, while a cell that is alive, but metabolically inactive, will have a lower degree of Cell Tracker™ Green CMFDA label visible in the cell. This low level Cell Tracker™ Green CMFDA labeling could be confused with a SYTOX® labeling if both vital stains were simultaneously added to the sample. The lack of a long pass emission filter on the UV filter set housed in the Lietz microscope precluded testing of the Cell Tracker™ Blue CMAC and SYTOX® Green combination. These two reporter fluorochromes are spectrally separated, and one would expect this combination of live and dead vital stains would be compatible, and distinguishable, in the same sample.

One concern in using vital stains on future samples collected from ballast test trials is the resulting status, or condition, of the cells following treatment. In the preliminary trials during this workshop, it was found that dead *Tetraselmis* cells purchased from Reed Mariculture had an inherent broad-spectrum autofluorescence that precluded the use of vital stains as it was impossible to determine if any dead or live stain was taken up within the cells. Upon realizing this problem, it was decided to use UV to irradiate live cells to kill them. These irradiated cells did not produce this broad-spectrum autofluorescence but rather had the expected chlorophyll *a* autofluorescence associated with freshly killed cells and allowed unambiguous stain labeling. Whether or not different ballast water treatment technologies will produce dead phytoplankon cells that have a broad-spectrum fluorescence is unknown and may be a function of a particular treatment type. As such, one must proceed with caution if vital stains are to be used to assess live and dead phytoplankton within these samples. To help account for broad-spectrum auto-fluorescence in algal cells, an unlabeled control should be prepared and examined using the filter sets that will be employed for vital stain analysis.

B.4 Moss Landing Marine Laboratory – Various Methods

It should be noted that this portion of the report was compiled by the NRLKW from disparate materials provided by MLML.

B.4.1 Flow Cytometry

A step-by-step protocol of the procedures used for MLML's flow cytometry work was not provided to NRLKW. It is known that MLML researchers used a Becton-Dickinson FACSort flow cytometer operated with CellQuest Acquisition Software and a CYTOWIN 4.31 analysis software package.

The settings used with the CYTOWIN 4.31 analysis software package were FSC: E-1, SSC 275, FL1 350, FL2 350, FL3 275 threshold FL3 100 with all voltages set in log mode. It is believed that these settings were used by MLML to classify detected particles as living cells, dead cells, or debris based on two fluorescence measurements and scatter measurements. With this information alone, it is impossible to assess the efficacy of these settings to distinguish the various phytoplankton classes. The settings are, however, only relevant to the specific flow cytometer, acquisition software and analysis software that was used by MLML to support the workshop.

The instrument was operated with a flow rate of 60 μL min^{-1} with data generally collected over 15 minutes per sample (some runs were shorter as a result of power failures). This implies that approximately 0.9 mL of sample volume were characterized by the MLML Flow Cytometer. In general, the group attempted to analyze 5 subsamples of both stained and unstained samples.

Stains were used at the following concentration levels by MLML:

- SYTOX® Green – 0.5 μM – incubation time 15 min in the dark – approximately 30 – 40 min to stain and process a subsample
- Calcein AM – 10 μM- incubation time 30 min in the dark – estimate 45- 60 min to stain and process a subsample.
- FDA – 10 μM- incubation time 30 min in the dark – estimate 45- 60 min to stain and process a subsample.

A review of the MLML flow cytometer data shows how the flow cytometer can be used in conjunction with vital stains (in this case SYTOX® Green) to distinguish both live and dead cells (Figure B-2). Again, it is unclear how the settings provided above are used to interpret these data and perform classification or how refinements in the software analysis parameters might impact classification results.

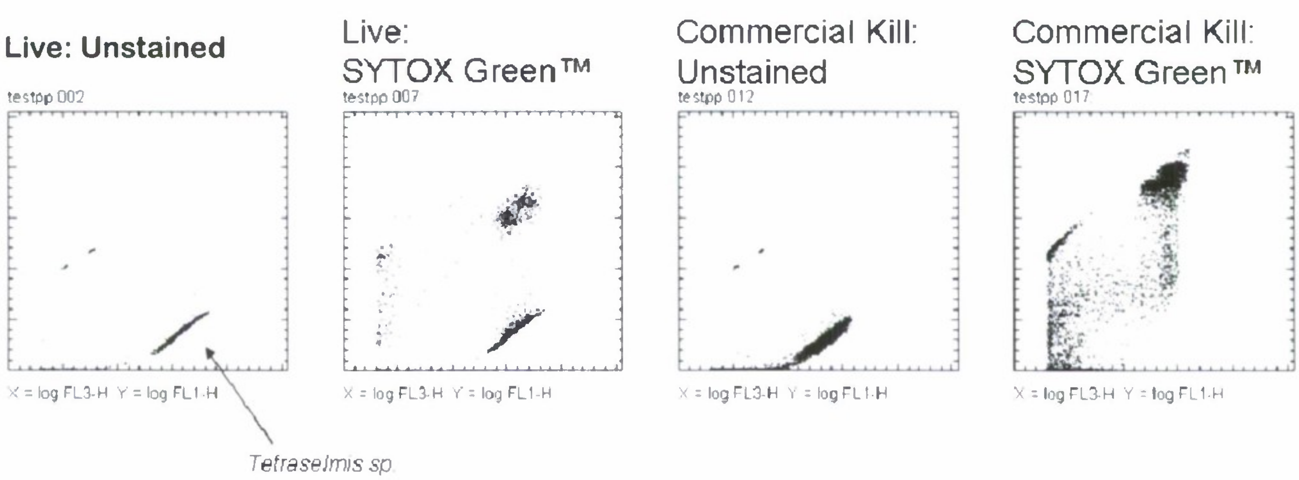

Figure B-2. Raw flow cytometry data from MLML.

Additional raw data from the MLML Flow Cytometer, shown in Figure B-3, were collected from the last, complex workshop sample that included suspended and dissolved solids. A review of the data indicates that because SYTOX® Green also stained the test dust particles, dead cells could not be easily identified using the flow cytometer; the dead cells' fluorescence signals overlap with test dust fluorescence signals (Figure B-3). The data also show when the samples are stained using FDA, the flow cytometer can be used to both identify the dead and live phytoplankton cells. Again, it is unclear how the analysis software settings are used to interpret these data and perform classification or how refinements in the software analysis settings might impact classification results.

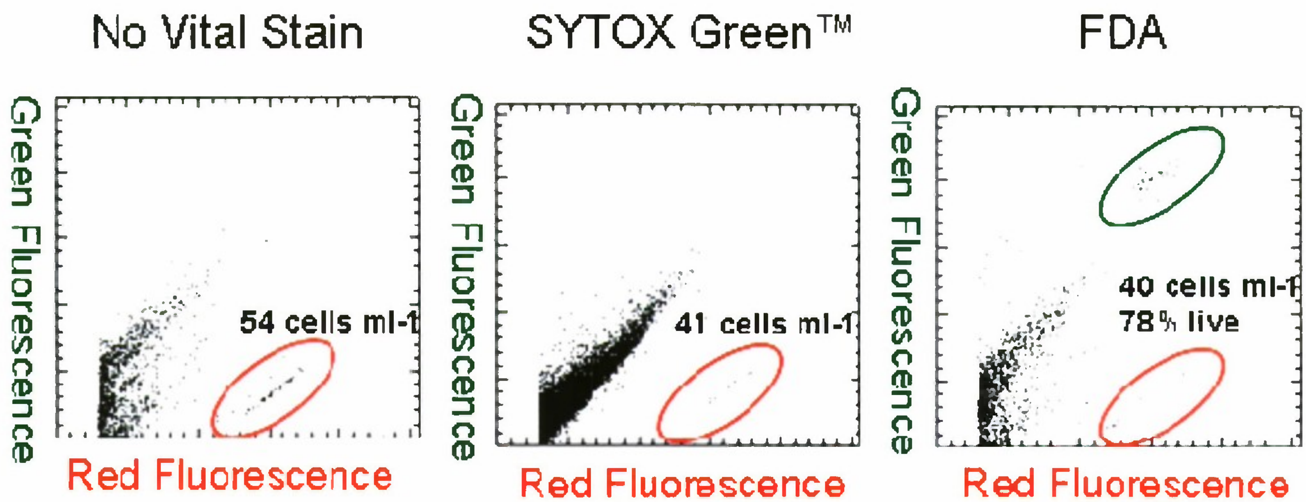

Figure B-3. Flow cytometry data from MLML showing improved live/dead detection with FDA in a workshop sample with added test dust.

B.4.2 Pulsed Amplitude Modulated (PAM) Fluorometry

This technique was used to evaluate the physiological condition of photoautotrophic organisms using a Walz Water-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The standard Walz 3 mL optical cuvet chamber was used only for samples of high cell density; growth media was used to dilute samples that were highly concentrated. This unit was not used with the workshop samples.

The workshop samples were evaluated using a Walz flow-through 350 mL cuvet system without dilution; the flow through system offers a ten-fold increase in sensitivity over the fixed cuvet configuration. The samples were kept in the dark until they were introduced in the cuvet systems under dim light, held for 3 minutes in the darkened cuvet, and analyzed with the PAM light-curve protocol. Values for the dark adapted determination of F_v/F_m are reported for many of the workshop samples.

B.4.3 Most probable number (MPN) determination of viable cell concentration

The MPN technique for determining viable cell densities is a culture-based method that relies on growth of target microorganisms monitored after quantitative serial dilution to extinction (that is, diluted to such a degree that no target cells are expected to grow in the most dilute series of subsamples). In concept, the technique can be applied to phytoplankton if suitably long incubations are employed to account for the slower growth rates of phytoplankton (about one doubling per day). We used whole cell fluorometric analysis on a sensitive SPEX Fluoromax 2 fluorometer to determine growth in fluid MPN cultures (3 mL)

incubated under continuous light and controlled temperature. Aliquots (3 mL) of undiluted samples were placed in each of 5 clear glass culture tubes. Each tube was then diluted serially five times (ten-fold dilution for each series) with fresh f/4 seawater media yielding a suite of 30 tubes, each filled to 3 mL (5 subsamples x 6 dilutions; relative dilutions were 1 through $1x10^{-5}$, in log increments). All culture tubes were identical and could be used as sample cuvets introduced directly in the fluorometer for fluorescence readings at excitation and emission wavelengths of 430 nm and 680 nm, respectively. MPN sample series were monitored every three days for three weeks to ascertain growth as indicated by fluorescence readings that were two-fold higher than the blank (filtered Barnstead NANOpure® water). The final MPN estimate of live cells mL^{-1} were obtained through computerized MPN tables for five subsamples.

MPN results were generated for three workshop samples and are presented in section 3.4.

B.4.4 Additional Measurement Methods

The following methods were also proposed by MLML for analyzing workshop samples. However, these methods were only applied by MLML to analyze highly concentrated samples of mostly live or dead cells that were produced by NRLKW for MLML. The methods are provided for completeness and because they are still potentially relevant to determining the concentration and viability of phytoplankton samples.

Photosynthetic rates: Carbon 14 Uptake (^{14}C) uptake

The ^{14}C technique provides a sensitive and simple means to evaluate bulk photosynthetic rates from the total phytoplankton community in any sample. A standardized incubation procedure was setup at MLML, California, where radioisotope facilities were available. Samples were shipped overnight from NRLKW to MLML for this purpose. Three shipments were received, each containing 'live' and 'dead' samples produced from two independent preparations made during the morning and afternoon preparation times at NRLKW. Two pairs of 'live/dead' samples were included in each shipment.

Samples, shipped with ice packs, were processed immediately upon arrival at MLML facilities. All ^{14}C incubations were made in triplicate, using 15-mL polypropylene centrifuge tubes as incubation vessels. Quantitative sample volumes (1-4 mL) were pipetted into the incubation vessels, filled to 14 mL with f/4 seawater media (to ensure nutrient saturation) and inoculated with quantitative additions of aqueous ^{14}C-bicarbonate solution, nominally 0.3 μCi (micro-curie) (Welschmeyer et al. 1991). The samples were placed on a rotating wheel exposed to 30 $\mu E\ m^2\ s^1$ irradiance from two fluorescent lamps; the incubation was made for 24 hours under continuous illumination in a 14 °C walk-in cold room. The samples were terminated by filtering the entire content of each vessel onto 25 mm GF/F filters. The filters were then fumed over HCl for 3 min to remove contaminate inorganic ^{14}C and were immersed immediately in scintillation vials containing 4-mL ScintiSafe* (Fisher Scientific, Pittsburgh PA) cocktail. Samples were counted on a Packard 2200L scintillation counter. The mean value of three incubation tubes filled with 14 mL f/4 media served as a blank that was subtracted from each subsample to compute final counts. A 50 μL aliquot from each blank tube was withdrawn prior to filtration and added directly to scintillation vials containing 4 mL NaOH-buffered scintillation cocktail to compute the total ^{14}C added to each sample. Specific activity (counts per minute per μgC) was computed assuming a total inorganic seawater concentration of 25 $mgC\ L^{-1}$.

The ^{14}C uptake measurements were not made on any of the mixed workshop samples. These measurements were made on highly concentrated samples of mostly dead or live phytoplankton. As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

Chlorophyll a determination

Chl *a* was determined fluorometrically using single-step extraction technique (Welschmeyer 1994). Samples were extracted in 90% acetone overnight at -4 °C; the extract was vortexed and diluted quantitatively with 90% acetone for readings taken on a Turner TD700 fluorometer fit with single-step, optical interference filters (Welschmeyer 1994). The fluorometer was calibrated with spectrophotometrically quantified pure chl *a* standards in 90% acetone.

As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

Adenosine triphosphate (ATP)

Measurements of particulate ATP were made using luciferin-luciferase based photometric assay (Karl 1980). Samples were harvested on 25 mm GF/F filters, extracted immediately in 4 mL boiling buffer (20 mM) and frozen until analysis on a Turner 20/20 ATP photometer. Reagents and ATP standards were supplied in a Promega Enlighten ATP assay kit.

As this method was not applied to any of the mixed samples, results from these measurements, although interesting, are not included in this report.

Method Advantages/Disadvantages

Even though the results are interesting, because of the lack of detailed information on the methods used on the workshop samples, it is not possible to list the advantages and disadvantages of the MLML methods.

B.5 NRLKW/Fluid Imaging Technologies –FlowCAM®

B.5.1 FlowCAM® System

NRLKW has been exploring the use of Fluid Imaging Technologies' FlowCAM® system (Figure B-4) as a means for automating the analysis of phytoplankton samples since 2004. Since that time, NRLKW has worked in conjunction with Fluid Imaging Technologies to implement the changes required in the FlowCAM® system to allow this instrument to be used for the automated analysis of phytoplankton samples from the BWTTF.

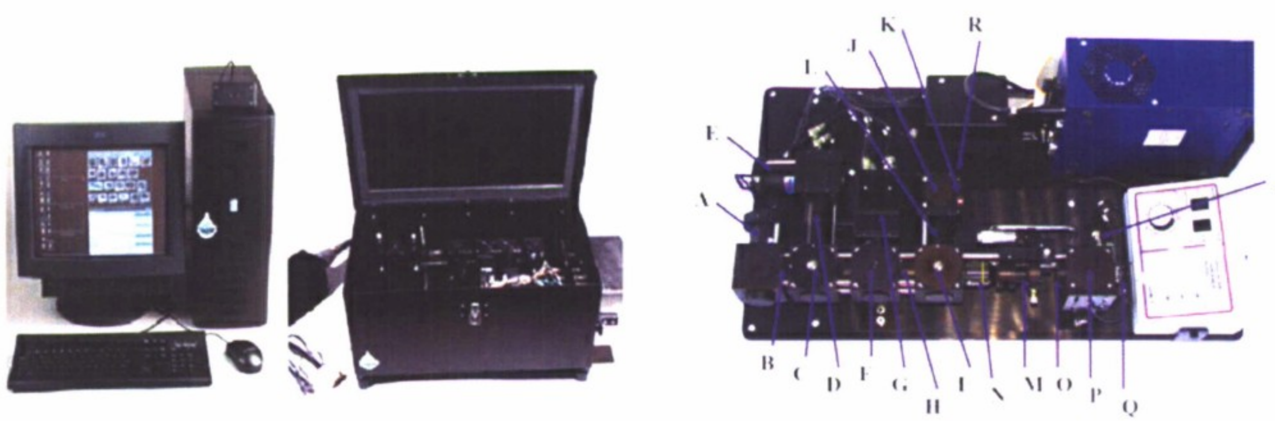

Figure B-4. FlowCAM® components and layout.

The FlowCAM® System and its components, depicted in the two photographs on the left of Figure B-4, consists of a PC used to control the system and to archive the collected data and a separately housed opto/mechanical breadboard. The breadboard itself is shown in the photograph on the right in Figure B-4. Housed on the breadboard are all the mechanical components required to bring the samples through a flow cell (pumps, controls, flow cell holder, etc.). Also on the breadboard are the optical components used to induce and measure florescence from particles passing through the flow cell as well as a detector that monitors particle-induced forward scatter from this same laser. When a particle is detected by either of the florescence channels or by the forward scatter channel, it is imaged as it passes through the flow cell. Imaging is accomplished using a unique optical configuration that uses a "flash" LED (light emitting diode) to illuminate the particle and a unique microscope objective/camera/custom optical element configuration that extends the microscope objective's depth of focus. As a result, this system produces very high-quality images of the detected particles.

During the course of working with the FlowCAM®, NRLKW has requested a number of modifications of the instrument that was initially purchased in 2004. These modifications include:

- Upgrade to a four-channel system (required for the simultaneous measurements of fluorescence at two wavelengths and forward scatter)
- Upgrade to a high resolution progressive video camera (improves the resolution of detected particles)
- Upgrade to a blue laser (required for compatibility with both DNA staining and metabolic dyes for viability assessment)
- Improve the sensitivity of the forward scatter channel (required as a result of the shift to a blue laser and a desire to have measurable forward scatter signals for phytoplankton sized particles).

It should also be noted that many of these modifications are now available in Fluid Imaging Technologies Systems.

The FlowCAM® produces a high-resolution image of each detected particle as well as useful florescence intensity signals (the first at the chlorophyll *a* autoflorescence wavelength and the second at a wavelength optimized for compatibility with the SYTOX® DNA staining dye) and a forward scatter intensity signal. The florescence and scatter intensity levels, in conjunction with image features generated by the FlowCAM® and additional features extracted from the high resolution imagery using in-house developed algorithms, offer the potential to fully automate the classification and enumeration of surrogate and indigenous phytoplankton and to determine their viability.

Work that NRLKW performed on the FlowCAM® in 2007 identified a serious problem with the instrument that would preclude its use for quantifying phytoplankton concentrations and viabilities in BWTTF samples [Nelson, Riley, Hogan, Lemieux (2007)]: when the two fluorescence channels and the forward scatter channels were operated simultaneously, all of the channel outputs became corrupted. The signal corruption was so great that dead cells would be classified as living ones based on the apparent lack of a SYTOX® fluorescence signal. The corruption also prevented the unit triggering on a forward scatter level appropriate for the reliable detection of phytoplankton.

NRLKW made Fluid Imaging Technologies aware of the issues with the NRLKW FlowCAM® unit. In turn, Fluid Imaging Technologies was able to verify that the problems identified with the FlowCAM® were endemic to all of the "Analog" versions of this system that had been produced up to that time (approximately 80 units). Fortunately, Fluid Imaging Technologies had been working on the development of a Digital Signal Processor (DSP) based version of the FlowCAM®, which uses a DSP instead of analog

circuitry to generate the fluorescent and scatter channel output signals. Initial experiments with the DSP-based FlowCAM® showed this technical approach obviated the problem inherent to the "Analog" version of the FlowCAM®.

Fluid Imaging Technologies was working on one additional change in the FlowCAM® that was of interest to NRLKW, that is, the design of a new type of flow cell to allow the entire width of the flow cell to be imaged. The original flow cells only allowed approximately 70 – 80 % of this width to be imaged. This restriction could create significant difficulties with the low cell concentrations that were anticipated in the Phytoplankton Enumeration and Evaluation Workshop sample matrix (as measurements are simply scaled to a higher value based on the flow cell's non-imaged area). The new flow cells were manufactured in a glass substrate that allowed their widths to be significantly reduced from the "free standing" flow cells that had been previously used with this system. At the time of the Workshop, prototypes of the new flow cell designs were available only in a size compatible with the 10X optical arrangement of the FlowCAM®. Most of NRLKW's previous work with phytoplankton had been done with the 20X optical arrangement of the FlowCAM®; however, the potential for missing phytoplankton cell as a result of the un-imaged areas of the original flow cell design presents a greater concern than the loss of spatial resolution that results from decreasing the FlowCAM®'s magnification from 20X to 10X. A new flow cell design that is compatible with the 20X optical configuration has become available from Fluid Imaging Technologies since the phytoplankton workshop.

It was desired to upgrade the NRLKW FlowCAM® unit prior to the start of the phytoplankton workshop. The NRLKW FlowCAM® had been modified multiple times in the past, and its configuration was not necessarily fully representative of the current systems being produced by Fluid Imaging Technologies. Further, the "analog" FlowCAM® could not be used to reliably determine if detected phytoplankton were viable, nor could it be known that the unit was triggering reliably on detected phytoplankton cells (as a result of issues with the forward scatter intensity measurements). Lastly, the lack of complete flow cell coverage with the FlowCAM®'s imaging system was a concern with the low concentration samples that were to be the emphasis of planned workshop measurements.

In November 2007, NRLKW and Fluid Imaging Technologies agreed to participate together in the phytoplankton enumeration experiments. As part of their participation in the workshop, Fluid Imaging agreed to update the NRLKW FlowCAM®. Specifically, the unit was first made optically (fully) and mechanically (mostly) identical to their current production units. The unit was upgraded to the DSP version, and the flow cell holder was modified to accommodate the new flow cells that were under development by Fluid Imaging Technologies. Fluid Imaging Technologies also agreed to fully train NRLKW to use the updated FlowCAM® and to perform initial experiments to demonstrate that the new version of the FlowCAM® overcame previous difficulties. Lastly, Fluid Imaging agreed to provide support for the first several days of testing associated with the Phytoplankton Enumeration Workshop. Researchers at NRLKW developed methods for determining cell viability using the FlowCAM® to be utilized during the workshop. Additionally, NRLKW personnel operated the FlowCAM® through all the measurements that were made during the phytoplankton enumeration experiment workshop. Through this arrangement, the NRLKW/Fluid Imaging Technologies team was able to make the current state of the art FlowCAM® available at the workshop.

It is important to note that the FlowCAM® was the only technique that was critically evaluated as part of this workshop. It is likely that an in-depth analysis and evaluation of any of the other workshop methods would indicate similar issues as those that are described in the following report sections.

B.5.2 FlowCAM® Methods

The following methods were used to process all but one of the Phytoplankton Enumeration Workshop samples. This method had to be modified to process the last workshop test sample because if its complexity (the addition of test dust, dissolved solids, etc.).

In order to determine cell viability, phytoplankton samples were first stained using SYTOX® Green prior to their introduction to the FlowCAM®. As is the case with the microscopic methods that have been previously described, SYTOX® Green stains the DNA in cells with compromised cell membranes. It is assumed that cells with compromised membranes are not viable. The addition of a SYTOX® Green to the sample creates some unique issues for the FlowCAM®. Autofluorescence of the SYTOX® Green in solution created a steady state background optical signal, which significantly reduced both the reliability and sensitivity of the photomultiplier tubes used in the FlowCAM® to detect both chlorophyll *a* and SYTOX® Green fluorescence.

Prior to the start of the phytoplankton enumeration experiment workshop, NRLKW used the following procedure to stain phytoplankton samples with SYTOX® Green: 10 µL of 5 mM concentration SYTOX® Green was first added to a 1 mL centrifuge tube. One mL of sample was next added to the aliquot, and the sample was mixed through multiple inversions. Figure B-5 shows a photograph of the FlowCAM® flow cell with a blank SYTOX® Green stained sample (e.g., a sample that contained 10 µL of 5 mM SYTOX® Green and 1 mL of artificial seawater). This type of photograph of the FlowCAM®'s flowcell was collected at the start of each run. The flow cell's entire width is visible, depicted by the white arrow, and the fluid's autofluorescent signal is clearly visible in the red ellipse (Figure B-5).

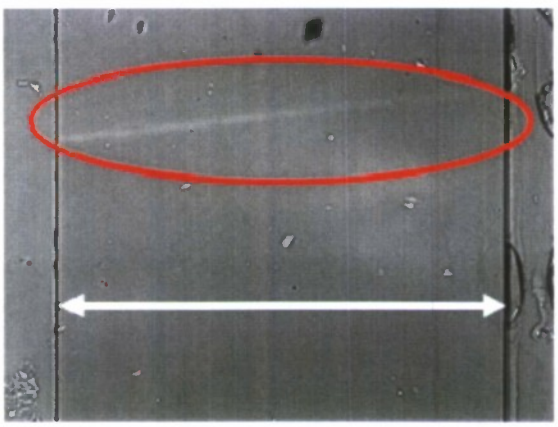

Figure B-5. SYTOX® Auto fluorescence in flow cell. with 10 µL of 5 mM SYTOX® added to 1 mL of artificial seawater.

The autofluorescence signal created a constant background signal that impacts the performance of the photomultiplier tubes used to detect fluorescence in the FlowCAM®. Discussions with other groups participating in the workshop that use SYTOX® Green to identify dead cells indicated the concentration of SYTOX® Green could be significantly reduced without reducing the stain's ability to detect dead cells (at least via manual microscopy or flow cytometry). Based on these recommendations, it was decided to reduce the concentration of the 10 µL of SYTOX® Green added to the 1 mL aliquots from 5 mM to 0.09 mM. Figure B-6 shows a photograph of the FlowCAM® flow cell with a blank SYTOX® Green stained sample using the lower concentration SYTOX® Green solution (e.g., a sample that contained 10 µL of 0.09 mM concentration SYTOX® Green and 1 mL of artificial seawater). Reducing the SYTOX® Green largely

eliminated the sample's auto-fluorescence signal (Figure B-6). It was also found (and substantiated by data provided later in this report) that reducing the SYTOX® Green concentration level did not impact the overall ability to stain and detect phytoplankton cells with compromised cell membranes. It is highly recommended that additional work be conducted to further explore the efficacy of SYTOX® Green staining over a range of lower concentration levels to fully optimize this concentration for detecting non-viable phytoplankton in solution.

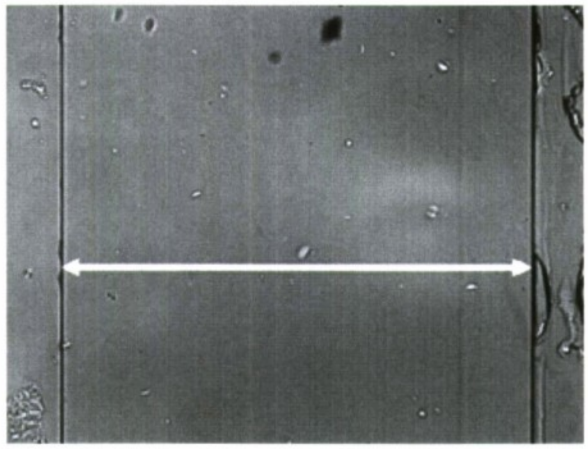

Figure 48. SYTOX® Auto fluorescence is not observed in the flow cell when 10 μL of 0.09 mM SYTOX® is added to 1 mL of artificial seawater.

Following the reduction of the SYTOX® Green concentration level, the following procedure was developed for working with the workshop's phytoplankton samples:

The pump on the FlowCAM® was first adjusted to a level that resulted in approximately one particle (cell, debris, etc.) situated in the instrument's field of view at a given time with the expected sample concentration levels. The field of view is defined approximately by the height of the image shown in Figures B-5 and B-6 by the white arrows. This optimum pump speed was empirically found to be approximately 1 mL 13 min^{-1} with the expected concentration levels of phytoplankton cells in the workshop samples. The pump speed was not adjusted during the entire Phytoplankton Enumeration Workshop. At this speed, with the exception of the last (more complicated) phytoplankton sample, multiple particles were rarely observed in the field of view simultaneously (< 5 % of the particles detected).

At the start of each day, a 1 mL SYTOX® Green blank was drawn through the flow cell to pre-condition the FlowCAM® prior to the introduction of the first real phytoplankton sample. The purpose of introducing this blank into the FlowCAM® was to obtain a representative background signal for the flow cell. This is important as the FlowCAM® may have detected a small background fluorescent signal that was not apparent in the photograph (e.g., Figure B-6) but which was picked up by FlowCAM®'s sensitive detectors. It should be noted that if the pump was turned off after the majority of the 1 mL blank sample was pumped through the flow cell, fluid remained in the system and obviated the need for any additional pre-conditioning of the FlowCAM®. The pump was typically turned off when the sample started to be pumped into the neck of the funnel used to introduce samples in the FlowCAM®. Data were collected and stored each day as the FlowCAM® was conditioned using the blank SYTOX® Green samples. Particles typically detected on these runs were a result of debris and salt crystals that did not fully dissolve in the artificial seawater. These types of particles were also encountered in phytoplankton samples.

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Following the conditioning of the unit with a SYTOX® Green blank (or with the SYTOX® Green blanks used to wash out the FlowCAM® following sample introduction), the instrument was started, and a 1 mL SYTOX® Green stained phytoplankton sample was introduced to the FlowCAM®. When the introduced sample approached the neck of the funnel used to bring samples into the FlowCAM®, a 0.5-mL SYTOX® Green stained artificial seawater sample was introduced to the FlowCAM®. The purpose of adding this blank sample to the FlowCAM® was to ensure all the cells from the sample were pumped through the flow cell. After this blank sample reached the neck of the funnel, a second 0.5-mL SYTOX® Green blank was introduced into the FlowCAM®. As in the case of the other 0.5-mL SYTOX® Green blank, the purpose of adding this was to ensure all the cells from the workshop sample were brought into and through the flow cell. When this last blank sample reached the neck of the funnel, the pump was stopped and the run terminated.

When the procedure outlined above was followed, it took approximately 30 min to process and collect the data associated with a 1 mL phytoplankton sample. Notably, it takes approximately the same time to process a 1 mL sample using a standard flow cytometer, such as the one used by the MLML. This is surprising because the flow cell in a flow cytometer is tapered such that only a single particle at a time is passes through the middle section of the flow cell, and the flow cell of the FlowCAM® is much wider than a single cell.

A standard method was also used to process the FlowCAM® data. Following the collection of data from a phytoplankton sample, the data were imported into the Fluid Imaging Technology Visual Spreadsheet® software, which was used to control and process data from the FlowCAM® (Figure B-7). The display provided four data plots as well as data on the particles that were detected during the run. The plot on the upper left shows the chlorophyll *a* fluorescence intensity (CH1 Peak) as a function of cell Equivalent Spherical Diameter (ESD). The ESD data were generated in this case from image processing operations performed on the particle image; they were not based on the amplitude of the forward scatter data, as they are in a standard flow cytometer.

The remainder of the plots are organized as follows: the upper right showed the SYTOX® Green fluorescence intensity (CH2 Peak) as a function of cell ESD; the plot on the lower left showed the forward scatter intensity (CH3 Peak) as a function of cell ESD; and the plot on the lower right provided the chlorophyll fluorescence intensity (CH1 Peak) as a function of the SYTOX® Green fluorescence intensity (CH2 Peak). It is this final plot in particular that was used when processing the phytoplankton data using the Visual Spreadsheet® software. There was also information regarding the particles that were detected. Most important were the total number of particles detected (737 in this case, Figure B-7). The particles mL^{-1} value indicated in the interface was not correct for the system as it was configured to support the phytoplankton workshop. This was a result of the new flow cell design and the operators not entering information to make this computation more meaningful. The time the run was started and stopped are also provided in the figure. Lastly, the figure provided information, including mean, range and standard deviation for many of the parameters that are extracted from each of the 737 detected particles. The information provided in this section of the software's graphical user interface (GUI) became more meaningful as sub-sets of the 737 detected particles were analyzed.

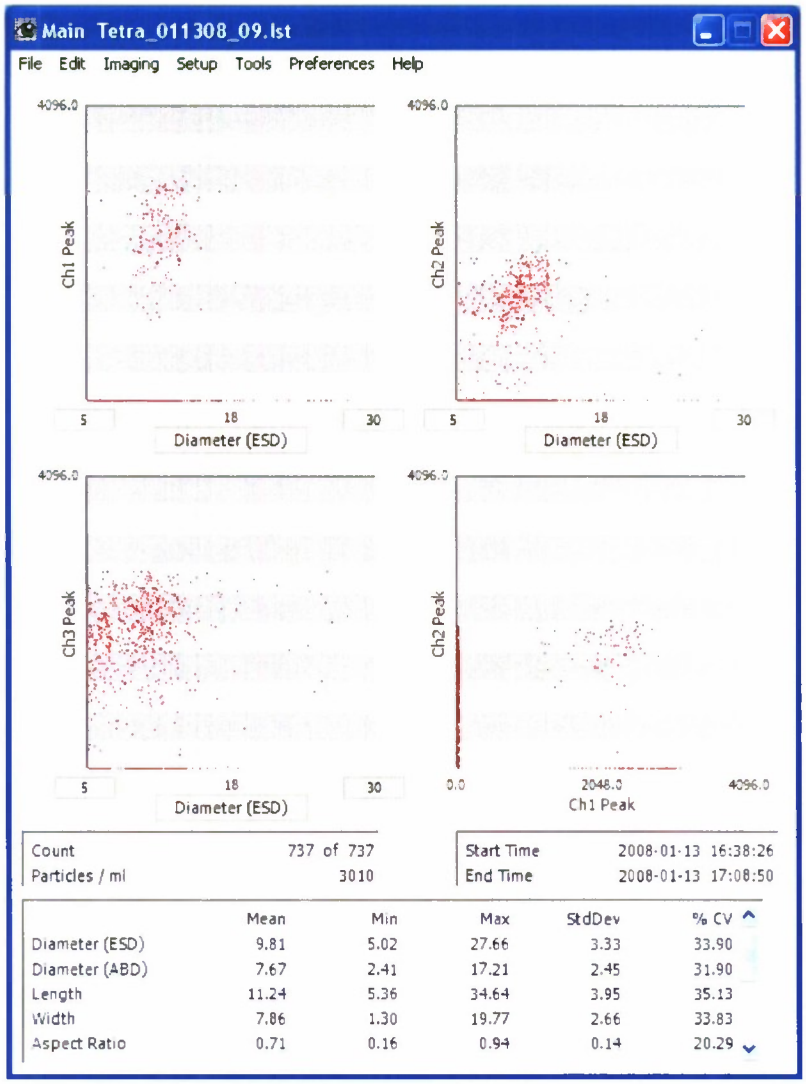


Figure 49. FlowCAM® GUI display for a workshop sample.

The first step in refining the analysis of the phytoplankton sample data was to identify all of the particles that have chlorophyll *a* but no SYTOX® Green fluorescence. This was accomplished by using the lower right plot and the Visual Spreadsheet® software to identify these particles. Using the software GUI, the operator first used a mouse to encircle the particles that have chlorophyll *a* but no SYTOX® Green fluorescence (Figure B-8; note that the GUI display was modified to only display the CH1 Peak vs. CH2 Peak plot). The ellipse along the CH 1 Peak axis identifies these particles, which are now highlighted in blue, and the display in the GUI now shows the operator has identified 95 of the 737 particles as having these properties. Next, the operator reviewed the images of the detected particles having these properties. The image on the right shows the 95 particles that exhibited chlorophyll *a* but no SYTOX® Green fluorescence (Figure B-8). Three particles of the 95 are highlighted in red. It is believed that these particles are not phytoplankton. In this case, all three of these particles were detected in the same field of view as phytoplankton cells. As such, the FlowCAM® automatically assigned them the same fluorescence and scatter values as the phytoplankton cell. This duality was indicated by the particle properties shown at the bottom of the figure. The bottom part of Figure B-8 shows two particles that were detected sequentially (particles 50 and 51) and that have the same CH1, CH2 and CH3 peak intensity values. The distribution of properties for the selected particles (bottom of left frame of Figure B-8) was much tighter than the

distribution for the total 737 particles (bottom of Figure B-7). The results of this initial analysis were that 92 particles were uniquely detected as having chlorophyll *a* fluorescence but not SYTOX® Green fluorescence and are therefore identified as viable phytoplankton.

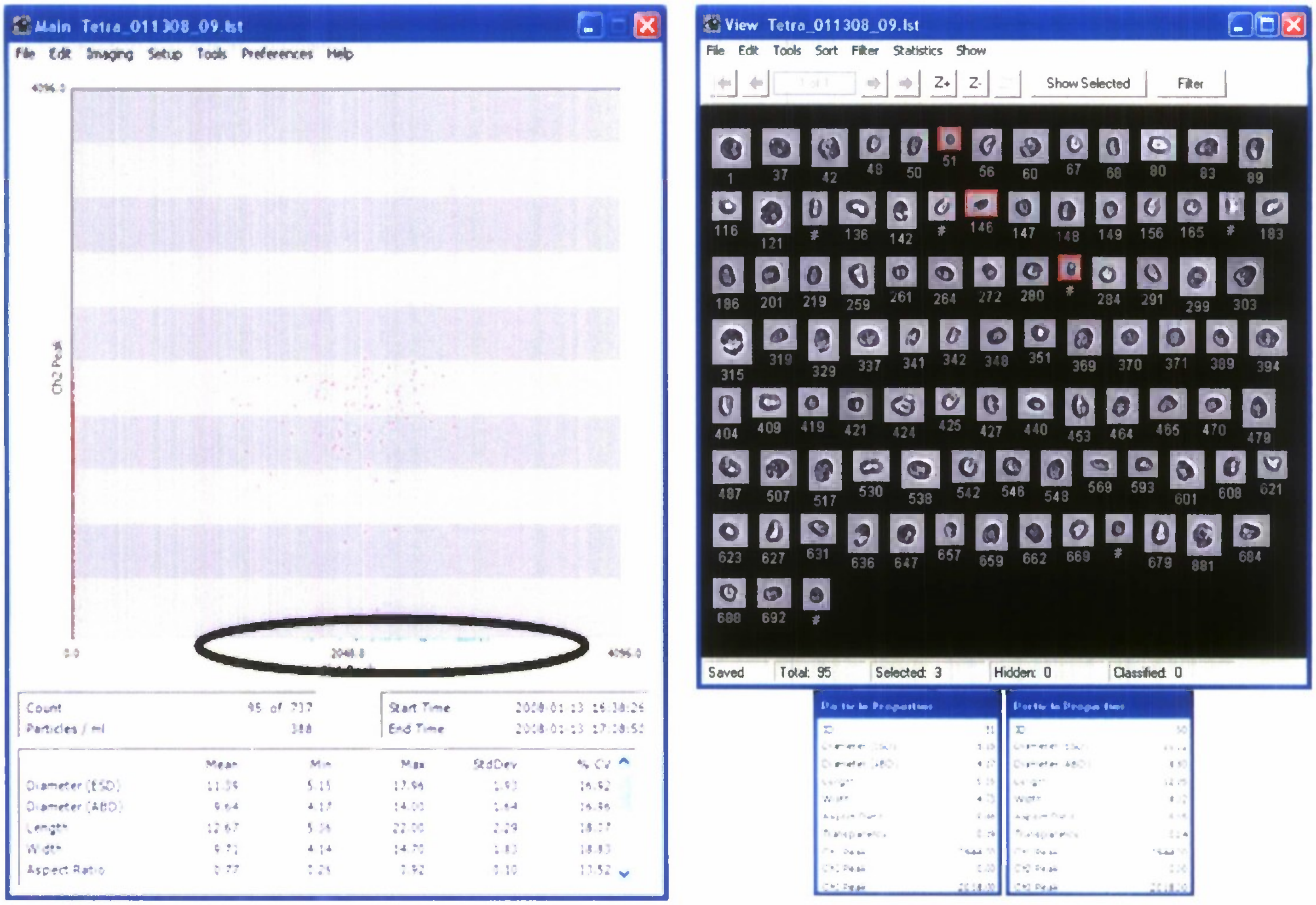


Figure B-8. Particles selected had chlorophyll *a* fluorescence (autofluorescence) but no SYTOX® fluorescence.

Next, the GUI was used to identify particles with both chlorophyll *a* and SYTOX® Green fluorescence. These were classified as non-viable cells. Using the same initial plot as before, the operator first used a mouse to encircle the particles that had both chlorophyll and SYTOX® Green fluorescence; the ellipse shown in the GUI identifies these particles, which are highlighted in blue (Figure B-9).

The operator then reviewed the images of the detected particles with these properties. The image on the right of Figure B-9 shows the 83 particles that exhibited chlorophyll *a* and SYTOX® Green fluorescence, with three particles highlighted in red because it is believed they are not phytoplankton. In this case, all three of these particles were detected in the same field of view as phytoplankton cells, which is evident after reviewing the individual particle attributes for the 83 detected particles. Note that the distribution of the selected particle properties shown at the bottom of the GUI interface (Figure B-9) is again much tighter than with the total 737 particles. The results of this initial analysis were that 80 particles were uniquely detected

having both chlorophyll *a* and SYTOX® Green fluorescence and were therefore identified as non-viable phytoplankton.

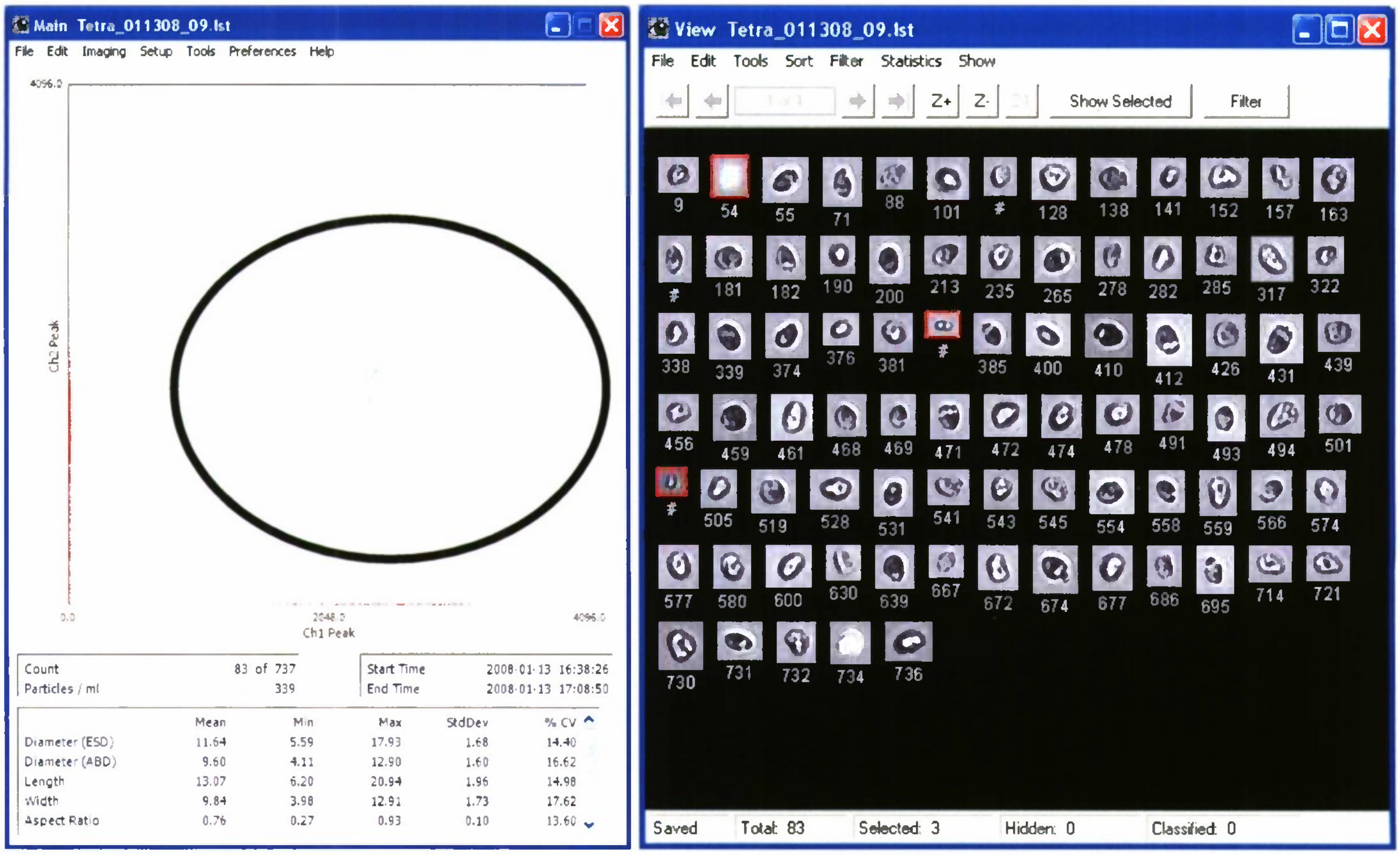


Figure B-9. Particles selected had chlorophyll *a* and SYTOX® fluorescence. These particles were classified as dead cells.

Because the current version of DSP-based FlowCAM® does not reliably measure chlorophyll *a* fluorescence intensity values for all detected phytoplankton, additional work needs to be performed to identify additional phytoplankton. (It is important to also note that this system does not measure forward scatter intensity values reliably. This statement is based on work performed with micro-beads (no signal) as well as many phytoplankton cells not producing a measurable forward scatter signal. It is also highly likely that the system does not measure SYTOX® Green fluorescence intensity values accurately based on the difficulties encountered with the other two channels.) To accomplish the task of identifying additional phytoplankton, the operator used the Visual Spreadsheet® software to highlight the detected particles that had measurable SYTOX® Green fluorescence intensity but no chlorophyll values (Figure B-10). The ellipse shown in the GUI identifies these particles (262 of 737 particles), which are now highlighted in blue (Figure B-10). Note the small region of red at the axes' intersection, which shows particles detected with no measurable SYTOX® Green or chlorophyll *a* fluorescence intensity values.

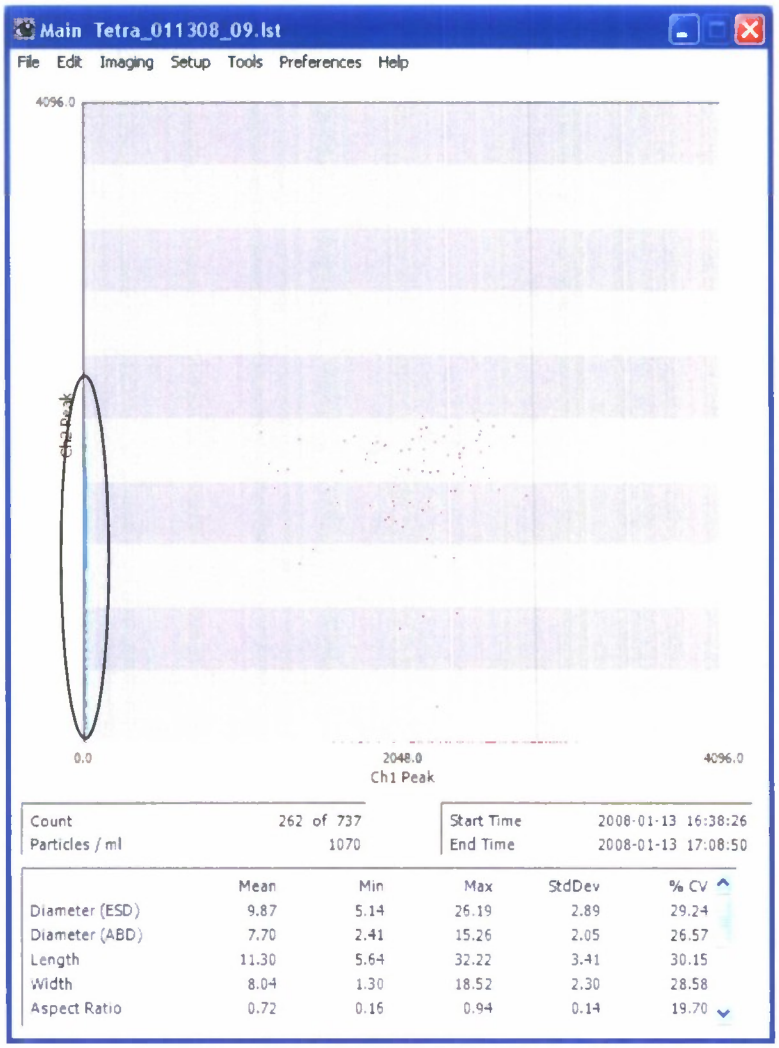


Figure B-10. Selected particles have SYTOX® Green but no chlorophyll *a* fluorescence. Some of these particles were classified as dead phytoplankton.

The 261 of the 262 particles with SYTOX® Green fluorescence are shown in Figure B-11. The operator next reviewed these images and highlighted (in red) the particles in the display that were likely phytoplankton. Here, 215 of the 262 detected particles appeared to be phytoplankton (Figure B-11). This manual classification is difficult because there is large variability in the properties of the phytoplankton in FlowCAM® imagery. It would be much better to corroborate this manual classification with a chlorophyll *a* fluorescence intensity level since such a coupling would provide greater confidence that all particles classified as phytoplankton in this process are truly phytoplankton and not dust or debris.

The results of the above analyses showed that 215 additional phytoplankton cells had measurable SYTOX® Green fluorescence. As such, these phytoplankton were classified as non-viable. Thus, the overall classification to this point was 92 viable phytoplankton mL^{-1} and 295 non-viable phytoplankton mL^{-1} (80 with chlorophyll *a* and SYTOX® Green fluorescence and 215 with only SYTOX® fluorescence).

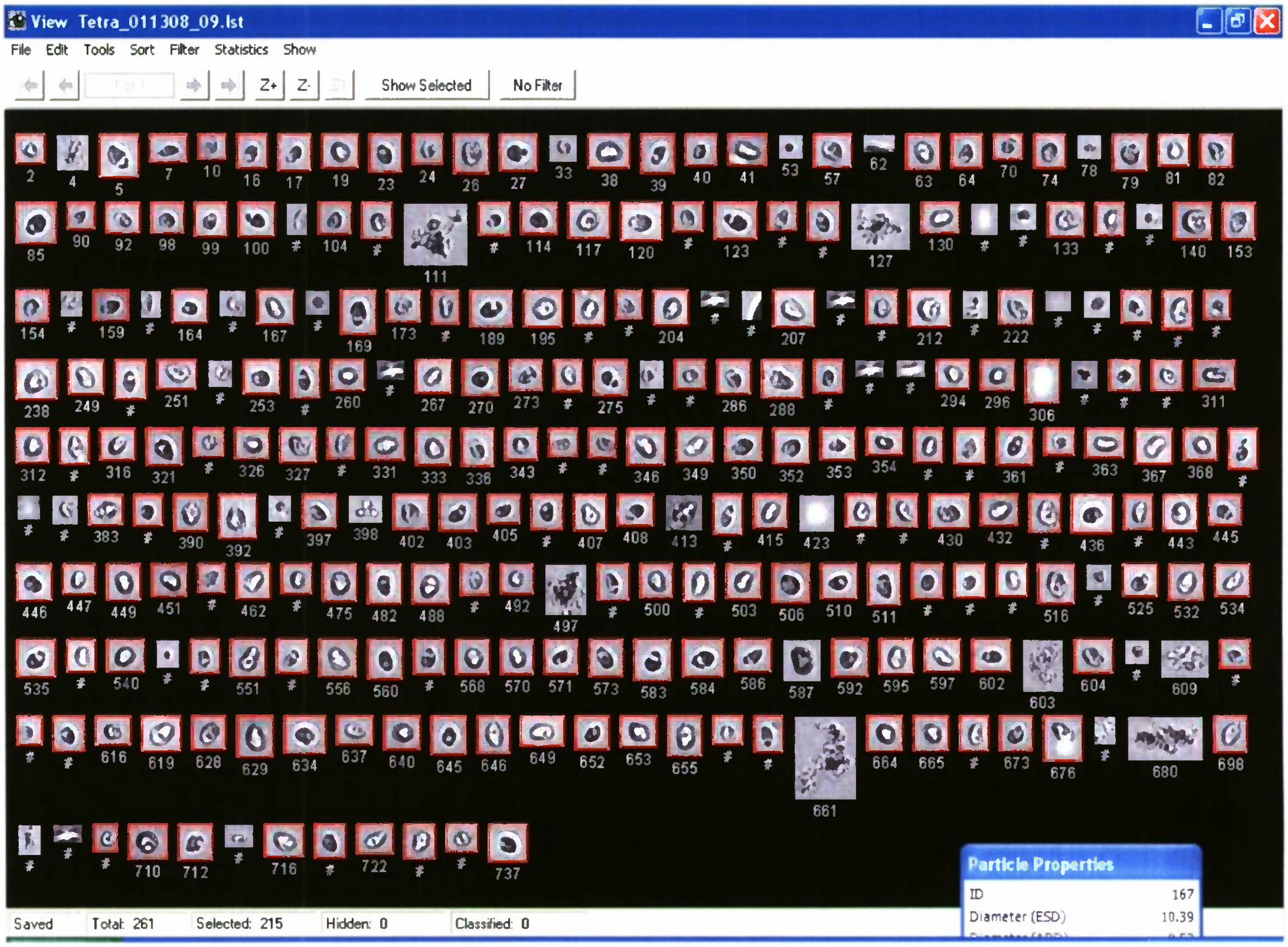


Figure B-11. Images associated with particles with SYTOX® fluorescence only. Following a review of the images, 215 of these 261 particles were classified as dead phytoplankton.

The remaining step in analyzing the FlowCAM® phytoplankton was to determine if any of the detected particles that did not have chlorophyll or SYTOX® fluorescence were, in fact, phytoplankton. As these particles did not have measurable SYTOX® fluorescence, the particles can not be classified as non-viable. That is, using this metric, they appeared to be viable. Because there are still major questions regarding the overall reliability of the Forward Scatter and chlorophyll *a* and SYTOX® fluorescence for phytoplankton sized particles, the particles were classified as phytoplankton strictly as a result of a manual review of the FlowCAM® images. Their viability was classified as unknown. As mentioned above, this process is complicated as a result of variability in the attributes of the phytoplankton in FlowCAM® imagery. It should also be noted that at least some of this variability was a direct result of physical differences in phytoplankton cells.

To analyze the particles that have neither chlorophyll *a* nor SYTOX® fluorescence, the Visual Spreadsheet® software was utilized with the operator using a mouse to encircle the particles of interest. The ellipse in Figure B-12 encircles 298 of the 737 particles with no measurable chlorophyll *a* or SYTOX® fluorescence (blue highlight).

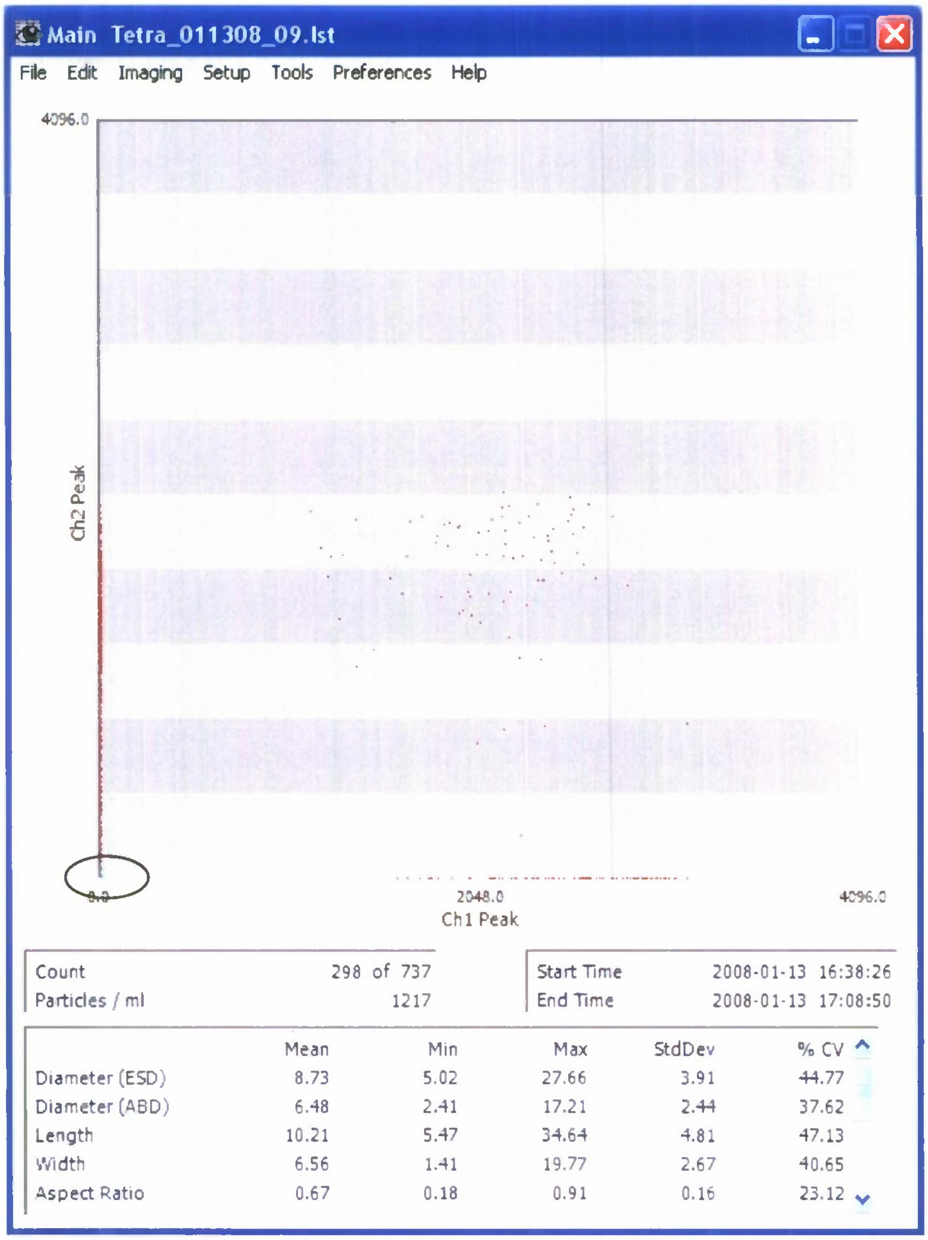
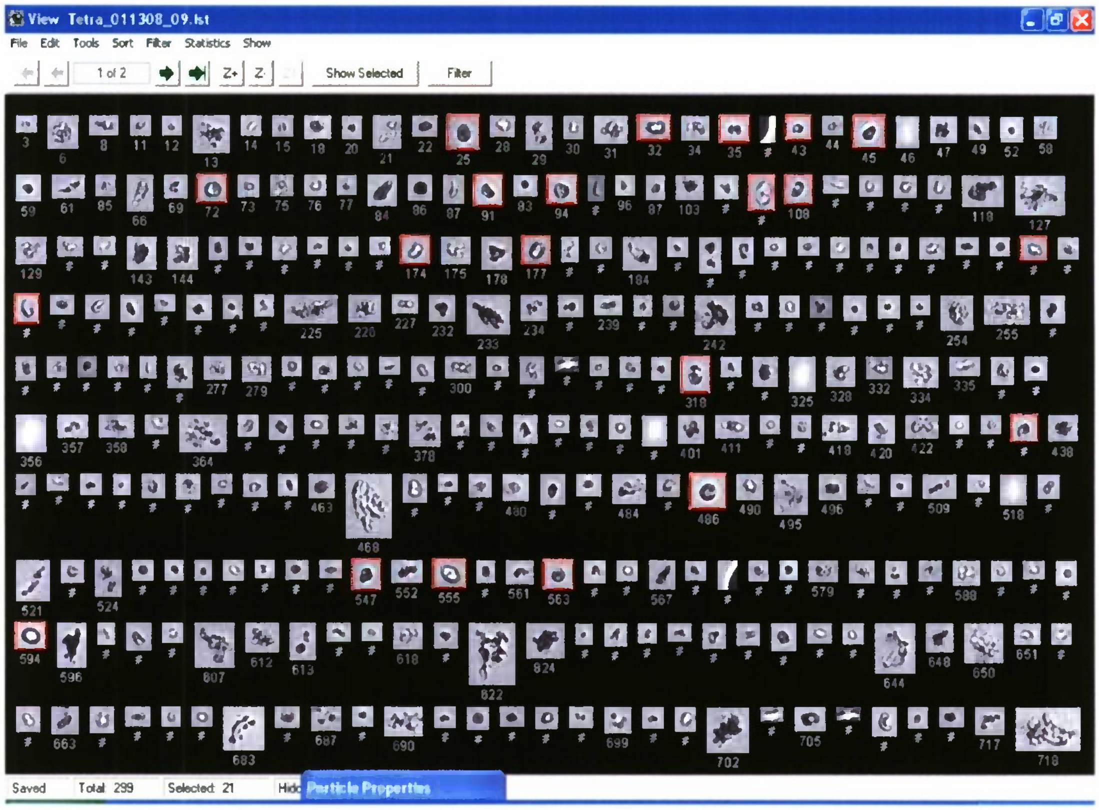


Figure B-12. Particles with no measurable chlorophyll *a* or SYTOX® Green fluorescence. Some of these particles were classified as cells of unknown viability.

The majority of the particles with neither chlorophyll *a* nor SYTOX® fluorescence are shown in Figure B-13. The operator next reviewed these images and highlighted (in red) particles in the display that were likely to be phytoplankton. The top panel of Figure B-13 shows the operator highlighted 21 phytoplankton-like particles. Because these particles were not assigned potentially erroneous fluorescent values, the Visual Spreadsheet® pattern recognition software can be used to identify additional particles that were similar to the ones that the operator specified (Note that erroneously measured forward scatter intensity levels creates difficulties for the pattern recognition software as the scatter values are used as a classification feature). The images identified as phytoplankton by pattern recognition software were next reviewed, and images selected by the software that appear *not* to be phytoplankton were removed from consideration (the bottom panel of Figure B-13). The combination of the manual review of the images and pattern recognition software data identified 13 additional phytoplankton cells.



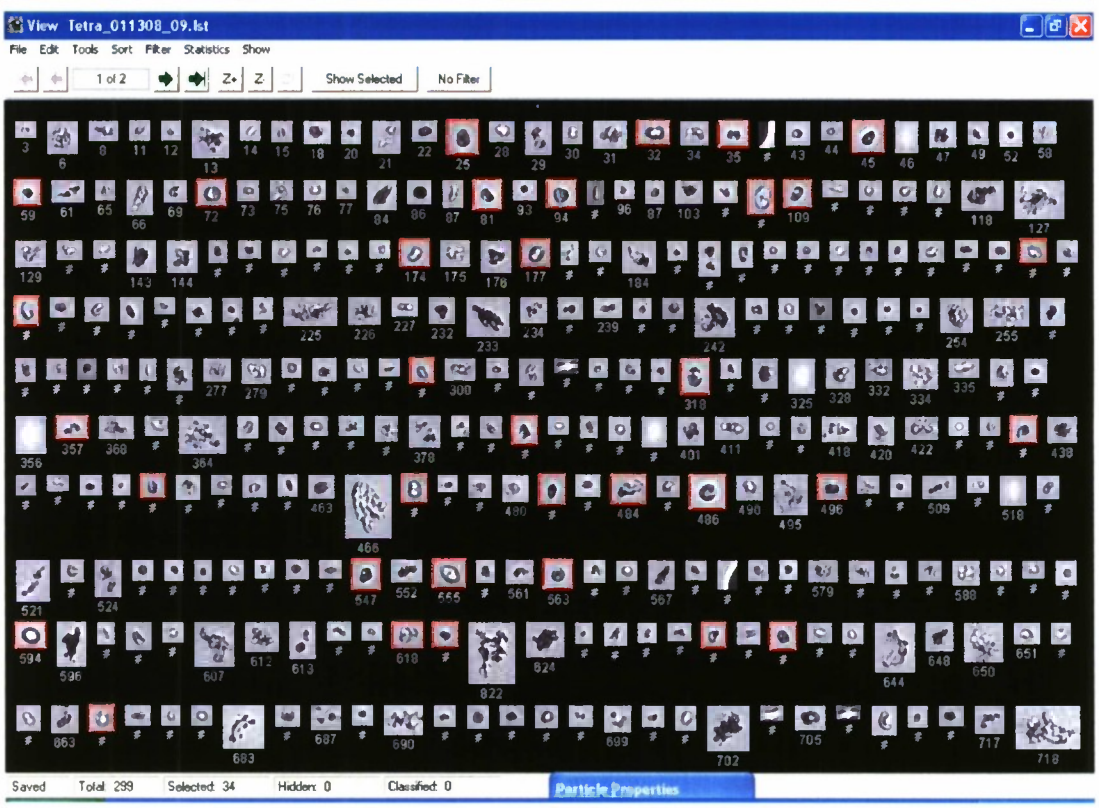


Figure B-13. Images of particles with no measurable SYTOX® or chlorophyll *a* fluorescence. Twenty-one particles were selected as potentially being phytoplankton cells in the upper image. The FlowCAM® software than selected 13 additional cells with similar properties

The 34 additional particles that were classified as phytoplankton are shown with more spatial resolution in Figure B-14. As was the case with the particles that exhibited only SYTOX® fluorescence, it would be beneficial to obtain corroborating data using chlorophyll *a* fluorescence value to verify these particles classified as phytoplankton did, in fact, contain chlorophyll *a*.

Figure B-14. Images of 34 particles that did not have chlorophyll *a* or SYTOX® fluorescence. These particles were classified as cells of unknown viability.

The final step required the operator to make two (subjective) assessments: how many additional particles with neither chlorophyll nor SYTOX® fluorescence might be phytoplankton? How many particles classified as phytoplankton might be debris? The results of these analyses (discussed above) indicated that 34 ±10 phytoplankton mL^{-1} were detected of unknown viability.

The overall results from this data set thus show:

- 92 viable phytoplankton mL^{-1}
- 295 non-viable phytoplankton mL^{-1}
- 34 ±10 phytoplankton mL^{-1} of unknown viability

The methods described in this section were applied to each sample analyzed during the Phytoplankton Enumeration and Enumeration Workshop, excluding the last sample analyzed. The last workshop sample was unique in that it had high concentrations of test dust and other suspended and dissolved particles in addition to the phytoplankton.

For the last sample, the above methodologies could not be applied. This was a direct result of the test dust that was added to the sample. All groups found that SYTOX® Green attaches to the test dust particles added to this last, complex sample. Further, the test dust was not neutrally buoyant. This creates two problems for the FlowCAM®: First, the stained test dust exhibited the same level of SYTOX® fluorescence as phytoplankton cells with compromised membranes (dead phytoplankton). This issue would present far fewer difficulties if the FlowCAM®'s chlorophyll fluorescence channel measured chlorophyll *a* fluorescence intensity values with consistency because unambiguous classification of phytoplankton and test dust requires that a measurable chlorophyll *a* fluorescence signal be measured for all detected phytoplankton cells. However, as previously described, there was no chlorophyll *a* fluorescence signal measured for over half of the total number of phytoplankton cells detected. Consequently, as the FlowCAM® was configured, the only method to separate SYTOX®-stained test dust from SYTOX®-stained phytoplankton cells was through visual inspection of the collected image sets. However, the spatial resolution of the images resulting from the 10X objective FlowCAM® configuration was not sufficient for performing this classification non-ambiguously.

The second problem resulted from the test dust being non-neutrally buoyant. Because the FlowCAM® used a vertical flow cell configuration and funnel for sample introduction, the non-neutrally buoyant test dust particles started to fall through the unit's funnel and into the flow cell immediately upon introduction. Thus, it was difficult to ensure that only a single particle was in the unit's field of view at a time. Furthermore, these particles fell through the column and into the test cell at a high rate at the start of each run and throughout regardless of the sample concentration and pump speed settings. Even with a 10:1 dilution of the test sample, on average, at least three particles were in the instrument's field of view each time the unit was triggered by a SYTOX®-stained test dust particle or when phytoplankton cells were detected through chlorophyll *a* fluorescence. As mentioned previously, when multiple particles were in the field of view at the same time, they were all assigned the same chlorophyll *a* fluorescence, SYTOX® fluorescence, and forward scatter intensity values. Thus, these metrics could not be used to reliably separate phytoplankton cells from test dust. When the unit was allowed to trigger with either the SYTOX® fluorescence and/or forward scatter channels active, the only method for determining whether the detected cells were phytoplankton was through manual review of the detected particles. Again, with the 10X objective configuration used in the FlowCAM®, it was not feasible to unambiguously separate phytoplankton signatures from those of test dust and other debris.

For the last sample, the original 1-mL test sample was diluted using 5:1 ratio based on the overall particle concentration levels anticipated with this sample. Five mL of sample were run through the FlowCAM®. Thus the total particle count for the sample was in units of detected particles mL^{-1}.

As a result of the above issues, the FlowCAM® was operated with just the chlorophyll *a* fluorescence channel active for the last, more complicated phytoplankton sample. This decision was made because operating with the SYTOX® fluorescence channel active resulted in the unit triggering at an unacceptably high frequency. This was also the case when the unit was operated with the forward scatter channel active. Further, whenever the unit was triggered by either of these two signals, there was an average of at least three particles in the instrument's field of view. The same was true using samples that were diluted by both 5:1 and 10:1 ratios. It is believed that this is a direct result of the inability to control rate at which non-neutrally buoyant particles travel through the FlowCAM®'s flow cell.

For the more complicated sample, three separate 5-mL samples were analyzed. As mentioned above, the unit was configured to only trigger on chlorophyll *a* fluorescence intensity level. Following the run, the images were manually analyzed to determine the total number of phytoplankton cells detected. As multiple particles were detected for each chlorophyll *a* fluorescence intensity level trigger, this was still a labor-intensive process. Because the unit had to be operated without the SYTOX® fluorescence channel active, it was also not possible to determine if the detected phytoplankton were viable or non-viable. Also, because the FlowCAM® did not measure a chlorophyll *a* fluorescence signal for more than half of the detected phytoplankton cells, it was likely that the cell concentration levels detected were lower than those actually in the test sample.

Consequently, for this last sample, only the total number of phytoplankton cells detected in the sample was reported. The number of viable and non-viable cells could not be determined for this sample using the FlowCAM®. The number of phytoplankton reported for this sample was the average of the number of cells detected in each of the three 5 mL samples that were analyzed.

B.5.3 Method Advantages and Disadvantages

The major advantage of the FlowCAM® is that it collects images of each particle it detects, thus allowing the operator to verify that detected particles are the result of phytoplankton cells of a particular type. Further, additional information from the detected particle can be extracted from the images using image processing methodologies. This could result in improved means for automating the classification of phytoplankton. It is important to note this capability is not provided in standard flow cytometers. Flow cytometer systems provide fluorescent signal intensity levels for each detected particle (both chlorophyll *a* and SYTOX®) and extract estimates of particle size (equivalent spherical diameters) from the forward scatter signal. They perform these measurements more accurately than the current configuration of the FlowCAM® because the flow cell geometry in flow cytometers results in these measurements being made on single particles always situated in the same position in the flow cell. There is no means, however, to verify that detected particles are truly from phytoplankton cells and that all detected phytoplankton cells are the same species with traditional flow cytometry.

There are several disadvantages of using the FlowCAM® in its current revision to analyze phytoplankton samples. First, the system still does not provide reliable and repeatable measurements of chlorophyll *a* and SYTOX® fluorescence intensity levels or forward scatter intensity levels. This inaccuracy makes it difficult to separate phytoplankton cells from other particles in the sample and also makes it much more difficult to automate the detection and classification (both type and viability) of phytoplankton. Fluid Imaging has recently replaced the forward scatter detector with a detector array which they claim has improved the reliability of the forward scatter detector. Additionally, they continue to work on improved measurement algorithms to enhance fluorescence detection. Unfortunately, these improvements were not implemented or available to the FlowCAM® that was used in support of this workshop.

Second, working with non-neutrally buoyant particles can be problematic with the FlowCAM® as a result of its flow cell configuration and its method for introducing samples to this flow cell. A flow cell that allows single particles to be interrogated would be advantageous, but it would likely significantly increase the time required to process a sample, since flow cells of this geometry would have significantly reduced flow rates and consequently slower sample throughput.

Third, a flow cell that allows the entire flow cell's width to be imaged was not available for use with the 20X objective FlowCAM® configuration. Using the FlowCAM® configured with a 10X objective compromised the spatial resolution of the particles' images and rendered it difficult—using either manual or automated methods—to unambiguously differentiate phytoplankton from test dust and other particles.

In response to the deficiencies mentioned above and in support of the Phytoplankton Enumeration and Evaluation Workshop, NRLKW and Fluid Imaging Technologies developed methods that helped obviate some of the deficiencies in the DSP based FlowCAM®. Because the "automated" and rapid analysis capabilities of the FlowCAM could not be utilized, significant manual analysis time was required to process the data. It is believed that the methodologies developed overcame the DSP based FlowCAM®'s deficiency in providing reliable and consistent fluorescence and forward scatter intensity measurements. If these measurements could be made more reliable, then it is likely that analysis of phytoplankton samples using the FlowCAM® could be performed more rapidly with high efficiencies.

The identified deficiency with the FlowCAM®'s current fluidic design and non-neutrally buoyant particles, however, should preclude its use in analyzing BWTE samples that have added mineral matter or other non-neutrally buoyant particles.

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APPENDIX C. ANOVA SUMMARY TABLES

The following tables summarize the results of the ANOVA analyses that were performed to compare the results obtained by each of the methods for the seven workshop samples. The purpose of performing these analyses was to determine if there were statistically significant differences between the measurement methods for each of the workshop samples.

The tables provide a summary of the ANOVA analyses for Live, Dead, and Total phytoplankton concentrations for each of the seven workshop samples. The charts in the Tables provide the mean values with error bars that indicate the 95 % confidence intervals for each of the workshop methods and for live, dead and total cell concentrations respectively. The 95 % confidence intervals are based on the pooled standard deviations. Also important in the table are the P values. A P value of less than 0.05 is indicative of statistically significant differences between the methods.

Terms used in the tables are defined as:

FCM S – MLML Flow Cytometry with SYTOX® Green

FCM C = MLML Flow Cytometry with FDA

Filtration C = WHOI Filtration with Cell Tracker™ Green CMFDA

Filtration S = WHOI Filtration with SYTOX® Green

Flowcam = NRL FlowCAM® with SYTOX® Green

SR = NRL Sedgwick Rafter with SYTOX® Green

CI = Confidence Interval

Pooled StDev = Pooled Standard Deviation

P = P value

DF = Degrees of freedom

SS = Sum of the Square Deviations from the Average

MS = Mean square (SS/DF)

F = F statistic (ratio of the two mean squares)

Table C-1. Measurement method comparison for workshop sample # 1.

```
Source     DF      SS     MS     F      P
Method 1    4   905.9  226.5  6.60  0.009              LIVE
Error       9   309.0   34.3
Total      13  1214.9


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level          N    Mean  StDev  -------+---------+---------+---------+--
FCM S          5   9.000  5.701  (-----*-----)
Filtration C   2  21.500  4.950          (---------*--------)
Filtration S   2  30.000  7.071                   (--------*--------)
Flowcam        2  20.500  0.707         (---------*--------)
SR             3  26.000  7.211                (-------*-------)
                                 -------+---------+---------+---------+--
                                       10        20        30        40
Pooled StDev = 5.859


Source     DF      SS     MS     F      P
Method 1    4    7198   1799  2.80  0.092              DEAD
Error       9    5788    643
Total      13   12986


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level          N    Mean  StDev  ---------+---------+---------+---------+
FCM S          5  137.80  19.56   (------*-------)
Filtration C   2  204.50  31.82                (----------*-----------)
Filtration S   2  155.00  19.80   (----------*-----------)
Flowcam        2  150.00  38.18  (-----------*----------)
SR             3  173.33  26.41          (---------*--------)
                                 ---------+---------+---------+---------+
                                        140       175       210       245
Pooled StDev = 25.36


Source     DF      SS     MS     F      P                  TOTAL
Method 1    4   11224   2806  3.42  0.058
Error       9    7381    820
Total      13   18605


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level          N    Mean  StDev  -+---------+---------+---------+--------
FCM S          5  146.80  18.82  (-------*------)
Filtration C   2  226.00  36.77                 (-----------*----------)
Filtration S   2  185.00  26.87       (----------*-----------)
Flowcam        2  193.00  48.08         (----------*-----------)
SR             3  199.33  28.10            (---------*--------)
                                 -+---------+---------+---------+--------
                                 120       160       200       240
Pooled StDev = 28.64
```

Table C-2. Measurement method comparison for workshop sample # 2.

LIVE

```
Source     DF      SS     MS      F      P
Method 2    4  3062.1  765.5  18.33  0.000
Error      10   417.5   41.8
Total      14  3479.6


                                   Individual 95% CIs For Mean Based on
                                   Pooled StDev
Level          N    Mean   StDev   --------+---------+---------+---------+-
FCM S          5  40.600   5.128    (---*---)
Filtration C   2  78.500   0.707                           (-----*------)
Filtration S   2  70.500  14.849                     (------*------)
Flowcam        3  41.667   2.887   (-----*----)
SR             3  53.333   6.110           (-----*----)
                                   --------+---------+---------+---------+-
                                          45        60        75        90

Pooled StDev = 6.462
```

DEAD

```
Source     DF     SS    MS     F      P
Method 2    4  30207  7552  3.67  0.043
Error      10  20588  2059
Total      14  50796


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level          N    Mean  StDev  --+---------+---------+---------+-------
FCM S          5  157.00  12.88   (------*-------)
Filtration C   2  192.50   3.54    (-----------*-----------)
Filtration S   2  180.50  26.16  (-----------*-----------)
Flowcam        3  257.67  88.52                (---------*---------)
SR             3  259.33  42.16                (---------*---------)
                                 --+---------+---------+---------+-------
                                 120       180       240       300

Pooled StDev = 45.37
```

TOTAL

```
Source     DF     SS     MS     F      P
Method 2    4  42391  10598  4.87  0.019
Error      10  21781   2178
Total      14  64172


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level          N    Mean  StDev  -----+---------+---------+---------+----
FCM S          5  197.60  13.01  (-------*-------)
Filtration C   2  271.00   4.24          (-----------*-----------)
Filtration S   2  251.00  41.01       (-----------*-----------)
Flowcam        3  329.33  86.00                    (---------*---------)
SR             3  312.67  48.01                 (---------*---------)
                                 -----+---------+---------+---------+----
                                    180       240       300       360

Pooled StDev = 46.67
```

Table C-3. Measurement method comparison for workshop sample # 3.

LIVE

```
Source      DF     SS    MS      F      P
Method 3     4  11740  2935  19.02  0.000
Error       13   2006   154
Total       17  13747


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level          N    Mean  StDev  ---+---------+---------+---------+------
FCM S          4   35.75   6.95    (----*-----)
Filtration C   3  100.00  14.42                          (-----*-----)
Filtration S   3   87.33  12.74                     (-----*-----)
Flowcam        5   58.80  13.94            (----*---)
SR             3   32.00  13.11  (-----*-----)
                                 ---+---------+---------+---------+------
                                   25        50        75       100

Pooled StDev = 12.42
```

DEAD

```
Source      DF     SS    MS      F      P
Method 3     4  35333  8833  28.03  0.000
Error       13   4096   315
Total       17  39429


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level          N    Mean  StDev  +---------+---------+---------+---------
FCM S          4   58.75   5.50          (---*---)
Filtration C   3   98.00  39.15                (----*---)
Filtration S   3   40.33   8.02      (---*---)
Flowcam        5   18.00   6.16  (---*--)
SR             3  144.67  18.15                         (---*---)
                                 +---------+---------+---------+---------
                                 0        50       100       150

Pooled StDev = 17.75
```

TOTAL

```
Source      DF     SS    MS     F      P
Method 3     4  22539  5635  7.76  0.002
Error       13   9436   726
Total       17  31975


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level          N    Mean  StDev  -------+---------+---------+---------+--
FCM S          4   94.50   9.95  (-----*-----)
Filtration C   3  198.00  52.20                      (------*-----)
Filtration S   3  127.67  19.66        (------*-----)
Flowcam        5  144.40  18.93             (----*----)
SR             3  176.67  27.23                  (-----*------)
                                 -------+---------+---------+---------+--
                                      100       150       200       250

Pooled StDev = 26.94
```

Table C-4. Measurement method comparison for workshop sample # 4.

```
Source     DF     SS    MS     F      P
Method 4    3  114.0  38.0  2.42  0.127                 LIVE
Error      10  157.2  15.7
Total      13  271.2


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level         N    Mean  StDev  -----+---------+---------+---------+----
Filtration C  3  13.667  0.577    (---------*----------)
Filtration S  3  14.667  0.577      (---------*----------)
Flowcam       5  11.600  5.505  (-------*-------)
SR            3  19.333  4.163               (----------*---------)
                                -----+---------+---------+---------+----
                                  10.0      15.0      20.0      25.0

Pooled StDev = 3.96


Source     DF      SS     MS     F      P
Method 4    3   76.89  25.63  2.63  0.108               DEAD
Error      10   97.47   9.75
Total      13  174.36


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level         N    Mean  StDev  ----+---------+---------+---------+-----
Filtration C  3   7.333  1.528     (-----------*----------)
Filtration S  3   6.000  1.000  (----------*-----------)
Flowcam       5  11.800  2.950                   (--------*--------)
SR            3  10.000  5.292             (-----------*----------)
                                ----+---------+---------+---------+-----
                                  3.5       7.0      10.5      14.0

Pooled StDev = 3.122


Source     DF      SS     MS      F      P
Method 4    3  2306.4  768.8  45.08  0.000              TOTAL
Error      10   170.5   17.1
Total      13  2476.9


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level         N    Mean  StDev  -----+---------+---------+---------+----
Filtration C  3  21.000  1.732   (----*----)
Filtration S  3  20.667  1.155  (-----*----)
Flowcam       5  49.600  3.975                              (----*---)
SR            3  29.333  7.024           (----*-----)
                                -----+---------+---------+---------+----
                                    20        30        40        50

Pooled StDev = 4.130
```

Table C-5. Measurement method comparison for Workshop sample # 5.

```
Source     DF      SS     MS     F      P
Method 5    5  2523.7  504.7  6.63  0.001             LIVE
Error      18  1370.1   76.1
Total      23  3893.8

                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level           N    Mean   StDev  --+---------+---------+---------+-------
FCM C           5  36.200  12.617             (----*-----)
FCM S           5  37.000   7.969             (-----*----)
Filtration C    3  26.667  13.503      (------*------)
Filtration S    3  55.000   1.732                        (------*------)
Flowcam         5  23.000   4.743     (----*-----)
SR              3  22.667   3.055   (------*------)
                                   --+---------+---------+---------+-------
                                    15        30        45        60

Pooled StDev = 8.725
```

```
Source     DF     SS    MS     F      P
Method 5    5   8815  1763  5.33  0.004                    DEAD
Error      18   5953   331
Total      23  14768

                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level           N   Mean  StDev  +---------+---------+---------+---------
FCM C           5  68.60  35.10                       (-----*------)
FCM S           5  48.60   7.50               (-----*------)
Filtration C    3  50.67   1.15             (--------*--------)
Filtration S    3  22.00   1.73  (--------*--------)
Flowcam         5  27.60  11.17      (------*------)
SR              3  77.33  12.06                     (--------*--------)
                                 +---------+---------+---------+---------
                                 0        25        50        75

Pooled StDev = 18.19
```

```
Source     DF     SS   MS     F      P
Method 5    5   4041  808  1.76  0.172                  TOTAL
Error      18   8268  459
Total      23  12309

                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level           N    Mean  StDev  -----+---------+---------+---------+----
FCM C           5  104.80  40.13                   (---------*---------)
FCM S           5   85.60   4.88          (---------*---------)
Filtration C    3   77.33  14.50   (------------*------------)
Filtration S    3   77.00   3.46   (------------*-----------)
Flowcam         5   70.40  15.36  (---------*---------)
SR              3  100.00  13.11              (------------*------------)
                                  -----+---------+---------+---------+----
                                      60        80       100       120

Pooled StDev = 21.43
```

Table C-6. Measurement method comparison for workshop sample # 6.

```
Source      DF      SS      MS       F       P
Method 6     5  154210   30842   86.40   0.000              LIVE
Error       18    6426     357
Total       23  160636


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level          N    Mean  StDev  -+---------+---------+---------+--------
FCM C          5   90.00   6.44          (-*-)
FCM S          5   95.60   8.79           (-*)
Filtration C   3  174.33  19.50                 (-*--)
Filtration S   3  300.00  46.78                             (-*-)
Flowcam        5   77.60  13.96         (-*-)
SR             3   16.67   4.16  (--*-)
                                 -+---------+---------+---------+--------
                                  0        100       200       300

Pooled StDev = 18.89


Source      DF      SS      MS       F       P
Method 6     5  681628  136326   59.40   0.000              DEAD
Error       18   41310    2295
Total       23  722938


                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level          N    Mean   StDev    ------+---------+---------+---------+---
FCM C          5  335.60   16.95        (--*--)
FCM S          5  318.00   37.07       (--*--)
Filtration C   3  780.33  114.09                               (---*--)
Filtration S   3  580.00   22.00                  (--*---)
Flowcam        5  261.60   39.68    (-*--)
SR             3  448.67   26.10             (---*---)
                                    ------+---------+---------+---------+---
                                         320       480       640       800

Pooled StDev = 47.91


Source      DF       SS      MS       F       P
Method 6     5  1129463  225893   83.93   0.000              TOTAL
Error       18    48444    2691
Total       23  1177907


                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level          N   Mean  StDev   ---+---------+---------+---------+------
FCM C          5  425.6   13.2     (-*--)
FCM S          5  413.6   40.1    (--*-)
Filtration C   3  954.7  133.6                            (--*--)
Filtration S   3  879.3   30.6                        (--*--)
Flowcam        5  394.0   22.9   (--*-)
SR             3  465.3   29.0      (--*--)
                                 ---+---------+---------+---------+------
                                   400       600       800      1000

Pooled StDev = 51.9
```

Table C-7. Measurement method comparison for workshop sample # 7.

```
Source     DF      SS     MS     F      P
Method 7    4  1027.3  256.8  3.19  0.046              LIVE
Error      14  1126.5   80.5
Total      18  2153.8


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level          N    Mean   StDev  --+---------+---------+---------+-------
FCM C          5  30.400   4.980  (------*-------)
FCM S          5  39.000  13.038         (-------*------)
Filtration C   3  37.333   6.429      (--------*--------)
Filtration S   3  35.000   5.568    (--------*--------)
SR             3  53.333  10.066                 (--------*---------)
                                  --+---------+---------+---------+-------
                                   24        36        48        60
Pooled StDev = 8.970


Source     DF      SS     MS     F      P
Method 7    4   728.5  182.1  4.63  0.014              DEAD
Error      14   550.1   39.3
Total      18  1278.6


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level          N    Mean   StDev  ------+---------+---------+---------+---
FCM C          5   8.800   4.087           (-------*-------)
FCM S          5  18.000   4.637                       (------*-------)
Filtration C   3   6.333   1.528      (---------*---------)
Filtration S   3   3.000   4.359  (---------*--------)
SR             3  19.333  13.317                    (---------*---------)
                                  ------+---------+---------+---------+---
                                       0.0       8.0      16.0      24.0
Pooled StDev = 6.269


Source     DF      SS     MS     F      P
Method 7    5  3430.9  686.2  6.96  0.001              TOTAL
Error      17  1676.9   98.6
Total      22  5107.7


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level          N    Mean   StDev  ---+---------+---------+---------+------
FCM C          5  39.200   8.289     (-----*-----)
FCM S          5  57.000  12.470                 (-----*-----)
Filtration C   3  43.667   5.132      (-------*-------)
Filtration S   3  38.000   9.165  (-------*-------)
Flowcam        4  37.250  10.275   (------*------)
SR             3  72.667  11.015                       (-------*--------)
                                  ---+---------+---------+---------+------
                                    30        45        60        75
Pooled StDev = 9.932
```